DEVELOPMENT OF RHIZOBIAL LIQUID INOCULANT PRODUCTION

Panlada Tittabutr

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การพัฒนาการผลิตหัวเชื้อไรโซเบียมชนิดเหลว

นางสาวพรรณลดา ติตตะบุตร

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การพัฒนาการผลิตหัวเชื้อไรโซเบียมชนิดเหลวจะต้องคำนึงถึงองค์ประกอบต่าง ๆ ที่ใช้ในการผลิต เช่น อาหารเลี้ยงเชื้อ วัสดุพาหะ และเชื้อไรโซเบียม ทั้งนี้เพื่อเป็นการลดต้นทุนการผลิตจึงได้นำวัสดุที่มีราคาถูกมาศึกษาเพื่อผลิตเป็นอาหารเลี้ยงเชื้อไรโซเบียม จากการศึกษาพบว่ามันสำปะหลังเป็นวัสดุที่สามารถผลิตน้ำตาลรีดิวซ์ได้สูงที่สุดเมื่อนำไปหมักกับเชื้อรา *Chlamydomucor* SUT1 โดยน้ำตาลรีดิวซ์ที่ได้จากการหมักมันสำปะหลังหนัก 100 กรัม (น้ำหนักสด) สามารถนำไปใช้เป็นแหล่งคาร์บอนเพื่อเลี้ยงไรโซเบียมได้ทุกสายพันธุ์ ยกเว้น *Bradyrhizobium japonicum* USDA110 อย่างไรก็ดีพบว่าเชื้อนี้สามารถเจริญได้ในอาหารที่มีกลีเซอรอลซึ่งได้จากกระบวนการหมักน้ำตาลรีดิวซ์ที่ได้ด้วยยีสต์ *Saccharomyces cerevisiae* F3109 ซึ่งเซลล์ยีสต์ที่ได้นี้ยังสามารถใช้ทดแทน Yeast extract ในสูตรอาหารได้อีกด้วย โดยสามารถเลี้ยง *B. japonicum* USDA110 ให้เจริญได้ถึง 3.61×10^9 เซลล์ต่อมิลลิลิตร จากนั้นทำการคัดเลือกวัสดุพาหะชนิดเหลวที่สนับสนุนให้หัวเชื้อไรโซเบียมมีอายุการเก็บรักษาได้นาน โดยทำการศึกษาวัสดุพาหะ 6 ชนิด เปรียบเทียบกับพีท (peat) ผลการศึกษาพบว่าเมื่อเก็บไว้ที่อุณหภูมิห้อง หัวเชื้อไรโซเบียมทุกสายพันธุ์ในวัสดุพาหะที่เป็นพีทมีอายุการเก็บรักษาได้นานที่สุด ในขณะที่อายุการเก็บรักษาของหัวเชื้อไรโซเบียมชนิดเหลวจะแตกต่างกันไปขึ้นอยู่กับสายพันธุ์ของไรโซเบียม และวัสดุพาหะที่ใช้ เมื่อทำการคลุกเมล็ดถั่วเหลืองกับหัวเชื้อไรโซเบียมแล้วเก็บไว้ที่อุณหภูมิ 40 องศาเซลเซียส เป็นระยะเวลา 48 ชั่วโมง พบว่าพีทสามารถรักษาปริมาณเชื้อไรโซเบียมให้มีชีวิตอยู่รอดบนเมล็ดได้มากที่สุด คือ 10^5 เซลล์ต่อเมล็ด และเมื่อใช้ arabic gum, sodium alginate, polyvinylpyrrolidone (PVP) และแป้งมันสำปะหลัง เป็นวัสดุพาหะในหัวเชื้อไรโซเบียมชนิดเหลวพบว่ามีปริมาณเชื้อไรโซเบียมมีชีวิตเหลืออยู่ 10^4-10^5 เซลล์ต่อเมล็ด ในขณะที่ polyethyleneglycol (PEG) และ polyvinylalcohol (PVA) เป็นวัสดุพาหะที่สามารถรักษาปริมาณเชื้อไรโซเบียมให้มีชีวิตอยู่รอดบนเมล็ดได้เพียง 10^3 เซลล์ต่อเมล็ด เมื่อทดสอบในสภาพไร่พบว่า หัวเชื้อไรโซเบียมชนิดเหลวทั้ง 6 ชนิด สามารถเข้าสร้างปม และให้ผลผลิตของถั่วเหลืองได้ดีเช่นเดียวกับการใช้หัวเชื้อไรโซเบียมที่ใช้พีทเป็นวัสดุพาหะ

การใช้หัวเชื้อไรโซเบียมคลุกกับเมล็ดถั่วเหลืองก่อนการปลูกนั้นทำให้เชื้อไรโซเบียมสัมผัสกับเมล็ดโดยตรง โดยสารที่หลั่งออกมาจากถั่วบางสายพันธุ์สามารถยับยั้งการเจริญของเชื้อไรโซเบียมที่อยู่บนเมล็ดได้ ดังนั้นเมื่อนำถั่วเหลืองสายพันธุ์ไทยจำนวน 5 สายพันธุ์มาทดสอบ พบว่าความสามารถในการยับยั้งการเจริญของเชื้อไรโซเบียมขึ้นอยู่กับสายพันธุ์ของถั่วเหลือง และความสามารถของเชื้อไรโซเบียมที่จะทนต่อสารยับยั้ง รวมทั้งปริมาณของสารที่หลั่งออกมาด้วย โดย

พบว่าสารที่หลั่งออกจากถั่วเหลืองอย่างน้อย 10 เมล็ด จึงจะทำให้เห็นผลการยับยั้ง โดยสารที่หลั่งออกมาจากถั่วเหลืองสายพันธุ์เชียงใหม่ 2 สามารถยับยั้งการเจริญของเชื้อไรโซเบียมได้มากที่สุด อีกทั้งยังพบว่าเซลล์ที่ไม่ได้ทำการล้าง exopolysaccharide (EPS) ออกไปนั้น มีความสามารถในการทนต่อสารยับยั้งที่หลั่งออกมาจากเมล็ดได้ดีขึ้น แสดงให้เห็นว่า EPS ที่ผลิตขึ้นโดยเชื้อไรโซเบียมเอง สามารถป้องกันเซลล์จากสารยับยั้งที่หลั่งออกมาจากเมล็ดถั่วเหลือง นอกจากนี้ยังเคยมีรายงานว่า EPS สามารถป้องกันเซลล์จากสภาวะแวดล้อมอื่น ๆ ที่ไม่เหมาะสมได้ ดังนั้นเพื่อทำให้เชื้อไรโซเบียมสร้าง EPS เพิ่มขึ้นโดยใช้วิธีการทางชีวโมเลกุล จึงได้ทำการศึกษาบทบาทของยีน *rpoH2* ต่อการควบคุมการผลิต EPS ในเชื้อไรโซเบียม สายพันธุ์ที่ใช้เป็นต้นแบบในการศึกษาคือ *Sinorhizobium* sp. BL3 ซึ่งเป็นสายพันธุ์ที่ได้สร้าง genomic DNA library ไว้แล้ว โดยคัดเลือกยีนส์นี้ด้วยวิธีการทำคอมพลีเมนเทชั่น (complementation) กับเชื้อ *Rhizobium* sp. TAL1145 *(rpoH2$^-$)* และเมื่อทำให้ยีนส์นี้ใน BL3 ไม่แสดงออกโดยการทำให้กลายพันธุ์ พบว่าเชื้อกลายพันธุ์ที่ได้ ไม่แสดงความแตกต่างของการผลิต EPS การ symbiosis และการเจริญในสภาวะที่มีอุณหภูมิสูง เมื่อเปรียบเทียบกับเชื้อ BL3 เดิม แต่ RpoH2 ที่ได้จาก BL3 สามารถทดแทนการทำงานของ RpoH2 ในการควบคุมการสร้าง EPS และการ symbiosis ใน TAL1145 *(rpoH2$^-$)* ได้ นอกจากนี้เชื้อไรโซเบียมควรจะมีความสามารถในการทนต่อสภาวะแวดล้อมตามธรรมชาติที่ไม่เหมาะสมอื่น ๆ อีก เช่น สภาวะดินที่เป็นกรดซึ่งพบได้ทั่วไปในประเทศไทย ดังนั้นในการพัฒนาเชื้อไรโซเบียม จำเป็นที่จะต้องศึกษากลไกที่ทำให้สามารถทน หรือเจริญในสภาวะที่ไม่เหมาะสมนี้ได้ ทั้งนี้พบว่า BL3 เป็นสายพันธุ์ที่สามารถพัฒนาตัวเองให้ทนต่อสภาวะที่เป็นกรดได้มากขึ้น เมื่อถูกเลี้ยงในอาหารที่มีความเป็นกรดต่ำกว่าแล้วเพิ่มขึ้นเป็นลำดับขั้นไป (Adaptive acid Tolerance Response; ATR) ดังนั้นจึงใช้สายพันธุ์ BL3 เป็นต้นแบบในการศึกษา โดยคัดเลือกคอสมิดที่มีชิ้นส่วนของยีนส์ที่เกี่ยวข้องกับ ATR ซึ่งอาศัยหลักการการเพิ่มการแสดงออกของยีนส์ผ่านทางจำนวนคอสมิดที่เพิ่มขึ้น แล้วถ่ายทอดเข้าไปสู่ BL3 พบว่า BL3 มีความสามารถในการเจริญในสภาวะที่เป็นกรดได้ดีกว่าเดิม จากนั้นตรวจสอบยืนยันโดยการทำให้กลายพันธุ์ ซึ่งในที่สุดพบว่ายีน *actX* ซึ่งสร้างโปรตีน histidine kinase sensor มีส่วนเกี่ยวข้องกับ ATR อีกทั้งพบว่าความสามารถในการเจริญในสภาวะที่เป็นกรดของเชื้อ BL3 ที่กลายพันธุ์ (*actX$^-$*) จะลดลงกว่าเดิม ในขณะที่เชื้อซึ่งเพิ่มการแสดงออกของ *actX* สามารถสร้างปมกับถั่วผี (*Phaseolus lathyroides*) ได้มากขึ้นในสภาวะที่เป็นกรด

สาขาวิชาเทคโนโลยีชีวภาพ ลายมือชื่อนักศึกษา พรรณลดา ศิวพรมงคล

ปีการศึกษา 2548 ลายมือชื่ออาจารย์ที่ปรึกษา

ลายมือชื่ออาจารย์ที่ปรึกษาร่วม

PANLADA TITTABUTR : DEVELOPMENT OF RHIZOBIAL LIQUID INOCULANT PRODUCTION. THESIS ADVISOR : PROF. NANTAKORN BOONKRED, Ph.D. 228 PP. ISBN 974-533-459-6

ACID TOLERANCE/CARRIER/INOCULANT/RHIZOBIA/SEED EXUDATES

To develop the formulation of rhizobial liquid inoculant, several components such as culture media, carriers, and rhizobia must be concerned. To reduce the cost of production, low-cost materials for culture medium production are important. By comparing several materials, it was found that cassava produced the highest amount of reducing sugar after fermentation with fungus (*Chlamydomucor* SUT1). All strains of rhizobia, except *Bradyrhizobium japonicum* USDA110, could grow well in the medium containing sugar derived from 100 g (wet weight) steamed cassava per liter. The derived sugar was further used for glycerol production by using yeast (*Saccharomyces cerevisiae* F3109). After fermentation, the formulated culture containing glycerol and heat-killed yeast cells, which might support as yeast extract supplement could promote bradyrhizobial growth up to 3.61×10^9 c.f.u./ml. In liquid inoculant production, 6 different carriers were investigated for their protective function with various strains of rhizobia compared with peat-based inoculant. Peat could promote long-term survival of all rhizobial strains, while the shelf-life of liquid inoculant was depended on strains of rhizobia and carriers, when stored at room temperature. Moreover, rhizobial survival in carriers after applied to seed under 40°C was also conducted and found that peat could maintain the highest number of rhizobial cell on seed at 10^5 cells/seed after stored for 48 h. Whereas, liquid inoculant containing arabic gum, sodium alginate, polyvinylpyrrolidone (PVP), cassava starch

could maintain at 10^4-10^5 cells/seed, and polyethyleneglycol (PEG) and polyvinylalcohol (PVA) could maintain only at 10^3 cells/seed. For nodulation test under field condition, it was found that all liquid inoculants showed equal performance as peat-based inoculant.

Since liquid inoculant must be contacted the seeds, it is important to determine whether the Thai soybean seed exudates affect growth of rhizobia. From five cultivars of soybean, CM2 showed the highest inhibition effect and the toxicity occurred only when exudates was derived from 10 seeds or more. The toxicity of exudates varied in soybean variety and the sensitivity of each rhizobial strain. However, there was no toxicity effect on the unremoved exopolysaccharide (EPS) cells. This indicated that the EPS naturally produced by rhizobia could protect itself from the toxicity of soybean seed exudates. The EPS has also known to protect cell from environmental stresses. Thus, it is interesting to increase EPS production in rhizobia by using molecular technique. *Sinorhizobium* sp. BL3 was used for this study since the genomic DNA library of BL3 has been constructed. To investigate the role of *rpoH2* in BL3 on EPS production, *rpoH2* gene of BL3 was isolated by complementation of *Rhizobium* sp. TAL1145 (*rpoH2*$^-$). Then *rpoH2* mutants of BL3 were generated by transposon mutagenesis. The *rpoH2* mutants of BL3 did not show significant difference on the EPS production, symbiosis, and the growth at high temperature when compared to wild-type. However, the clone containing *rpoH2* gene of BL3 could restore both EPS production and nodulation defects of TAL1145 *rpoH2* mutants. Besides EPS mechanism to protect toxic substances, inoculated rhizobia should also survive in acidic condition which is the most common found in Thai soil. To improve rhizobial strain, it is necessary to determine the mechanism of rhizobia in order to well

establish in soil acidity condition. Since BL3 showed the property of adaptive acid tolerance response (ATR), thus rendering used as a model for this study. The gene involved in ATR was identified on the basis of enhancing its expression through increasing its copy number in acid sensitive strain. It was found that *actX* gene encoded for a histidine kinase sensor protein is involved in ATR. This was also identified and confirmed by transposon mutagenesis. The *actX* mutant of BL3 performed the increment of acid sensitivity. Inoculation of *Phaseolus lathyroides* seedlings with the complemented mutant containing multiple copies of *actX* was able to enhance nodulation efficiency at low pH.

School of Biotechnology

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Student's Signature_____

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Co-advisor's Signature_____

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Panlada Tittabutr

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LIST OF ABBREVIATIONS

Act	=	protein involved in acid tolerance
ATR	=	adaptive acid tolerance response
bp	=	base pair
c.f.u.	=	colony forming unit
ChvI	=	response regulator involved in regulating EPS I biosynthesis
DNA	=	deoxyribonucleic acid
DO	=	dissolved oxygen
EPS	=	exopolysaccharide
Exo	=	protein involved in succinoglycan (EPS I) biosysthesis
Exp	=	protein involved in galactoglucan (EPS II) biosynthesis
ha	=	hectare
kb	=	kilobase pair
kDa	=	kilodalton
M	=	molarity
mM	=	millimolarity
MucR	=	transcriptional repressor of galactoglucan biosynthesis
Nod	=	bacterial protein involved in legume nodulation
Ntr	=	nitrogen regulatory protein
OD	=	optical density
PCR	=	polymerase chain reaction
pHe	=	external pH

LIST OF ABBREVIATIONS (Continued)

pH_i	=	internal pH
Pho	=	phosphate transcriptional regulatory protein
RNA	=	ribonucleic acid
Ros	=	transcriptional repressor of *Agrobacterium* virulence genes
Rpo	=	RNA polymerase subunit protein
Syr	=	symbiotic regulatory protein
U	=	unit

CHAPTER I

INTRODUCTION

The overall goal of this research is to develop improved culture media and carrier material for enhancing survival of rhizobial inoculants. Although there are well-defined culture media currently available for growing rhizobia, these formulations use expensive ingredients, some of which are not locally available in Thailand. Therefore, it should be possible to improve culture media for rhizobia using locally available material. Similarly, there is a need to develop alternative forms of carrier to peat that ensures the maintenance of inoculant quality during storage and transport to the field. Rhizobial inoculants are often exposed to environmental stresses during storage and immediately after application to the seed. Since the success of rhizobial inoculation depends on maintaining rhizobial viability, it is important to understand how environmental stresses affect rhizobial survival.

Soybean is the most important grain legume in the world occupying 78.9 million ha during year 2001-2002 (Agbioforum, www, 2005). In Thailand, soybean occupied 2,118 ha with an annual production of 292,098 tons during year 2003-2004 (DOA, www, 2005). In 2000, Thailand imported 660,000 tons of soybeans from other countries (Foodmarketexchange, www, 2005). The productivity of soybean in Thailand can be increased by expanding the acres under soybean and by applying *Bradyrhizobium* inoculants. Use of *Bradyrhizobium* inoculant instead of chemical fertilizer has been shown to reduce the cost of soybean production. Boonkerd (2002)

investigated the soybean productivity and economic gain due to different cultural practices and observed more than 50% increase in soybean yield due to *Bradyrhizobium* application (Table 1.1). It was found in that experiment that 1,250 g of inoculant could replace 179.2 kg of urea fertilizer, or 200 g of *Bradyrhizobium* inoculant for 28.6 kg of urea. These results demonstrate that *Bradyrhizobium* inoculation is necessary for enhancing soybean yield in Thailand. Numerous other studies in many other places of the world have also shown that *Bradyrhizobium* inoculant can increase soybean yield (Catroux, 1991), and thus providing higher net benefit than using chemical fertilizer.

Table 1.1 Soybean productivity and economic gain due to rhizobial inoculant application

Treatments	Yield (kg/ha)	Net benefit over control (US$/ha)
Control (without rhizobial and fertilizer)	803	0
Rhizobial inoculant	1,228	126.7
Rhizobial inoculant + P (56.3 kg/ha) + K (37.5 kg/ha)	1,352	109.7
N : P : K (75 : 56 : 37 kg/ha)	1,224	26.5

Modified from Boonkerd (2002)

Inoculation of the entire area under soybean in Thailand with *Bradyrhizobium* requires efficient inoculant production, storage and distribution system. For production of *Bradyrhizobium* inoculant, a low-cost medium that uses locally available material is preferable. Currently available culture media use yeast extract and mannitol as essential ingredients. It should be beneficial if these ingredients can be substituted with a locally-available material. Following production, the inoculant has to be stored and transported to the field throughout Thailand where soybean is cultivated. Currently, peat-based carrier material is used for storage and transport of inoculants to distant places (Somasegaran and Hoben, 1994; Roughley and Vincent, 1967; Feng et al., 2002). Also most of the rhizobial inoculant produced in Thailand uses peat dust as the carrier (Boonkerd, 2002). The material for peat-based carriers is obtained from a naturally-occurring organic material. The supply of such peat-based organic material is also limited. Even other solid materials, such as lignite, charcoal, coir dust, and compost of various agricultural wastes have been used instead of peat, but their performance characteristics are not equivalent to peat-based inoculant products (Singleton et al., 2002). Therefore it is important to develop alternative carrier material for rhizobial inoculant production.

Rhizobium inoculants are usually mixed with seeds just before sowing in the field. Mixing of inoculant with seeds ensures placement of rhizobia close to the germinating seed, thus facilitating infection and nodule development. This also helps to uniformly distribute rhizobia over the entire field. However, it is often difficult to uniformly mix peat-based inoculant with seeds. Solid-based inoculant also tends to plug precision air seeders (Singleton et al., 2002). Because of these difficulties inoculant producers have been changing to liquid inoculant formulations instead of

solid-based inoculants. Liquid inoculant can be prepared in broth culture or as frozen concentrate, and conveniently mixed with water for spraying into seed furrow or coating directly onto the seed without using sticker (Brick, www, 1999). Moreover, liquid inoculant coats seed uniformly and dries when applied through a seed auger. Seeds coated with liquid inoculant flow well when planted by using various types of seeding equipment. Soluble materials that promote survival of rhizobial in the inoculant and after application to seed are usually liquid formulations (Vincent, 1958, quoted in Deaker et al., 2004; Heller, 1941; Lloyd, 1979).

Research described in this thesis attempts to identify and evaluate new liquid inoculant additives that can be added to culture medium which allow normal cell growth, supports high cell number during storage, and promotes nodulation better than or equivalent to peat-based inoculant.

There have been many reports about the presence of toxins in leguminous seed coats, including soybean seed. Rhizobia on the seed surface of these legumes show reduce viability (Materon and Weaver, 1984; Bowen, 1961; Young and Peterson, 1980; Singleton et al., 2002). To use liquid inoculant for Thai soybean varieties, it is important to determine if seeds of these varieties exude any chemicals that may be toxic to rhizobia.

Rhizobial inoculant is often exposed to unfavorable stresses when inoculated seed are planted in the soil. These stresses include such things as low or high pH, high temperature, low moisture and high salt concentrations. Therefore, the rhizobia in inoculant should be tolerant to these stresses, have protecting materials in the inoculant formulation or have adaptive mechanisms that increase their tolerance.

Adaptive tolerance is a homeostasis mechanism that allows bacteria to survive and grow after sudden changes in environmental conditions. In the northeastern region of Thailand, the soil pH is generally low (pH 5.0-6.0) (SWU, www, 2005). Therefore, the rhizobial inoculant for this region should have adaptive tolerance response to low pH conditions. Rhizobial cells are surrounded by a layer of exopolysaccharide (EPS), which has been reported to have a role in protecting cells under stress conditions, such as exposure to toxic elements and desiccation by slowing the rate of drying within colony microenvironment (Hatel and Alexander, 1986; Roberson and Firestone, 1992; Ophir and Gutnick, 1994). Therefore, EPS production by the rhizobial cell itself could be the one of strategies that protects inoculated rhizobial cells from stress factors.

1.1 Research objectives

(i) To develop a low-cost rhizobial growth medium production with locally available material.

(ii) To investigate new additives to liquid formulations that can improve cell survival, and provide field performance equal to or better than peat based inoculant.

(iii) To determine whether Thai soybean seed exudates affect growth of rhizobia.

(iv) To identify mechanisms and their genetic control that allows rhizobial to grow and survive particular environmental stress.

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CHAPTER II

REVIEW OF LITERATURES

Rhizobia is soil bacteria, which including genera *Rhizobium*, *Bradyrhizobium, Sinorhizobium, Mesorhizobium, Allorhizobium,* and *Azorhizobium*, which are able to symbiosis with leguminous plants. They could form the specialized organs, called nodules, on roots or stems of their hosts. Rhizobia inside nodule could reduce atmospheric nitrogen and make it available to the plant. Symbiotic nitrogen fixation is an important source of nitrogen, and various legume crops could fix as much as 200 to 300 kg of nitrogen per hectare or about 70 million metric tons of nitrogen per year (Peoples et al., 1995; Brockwell et al., 1995). Then, biological nitrogen fixation can be an important component of sustainable agricultural systems, and using instead of chemical nitrogen fertilizer, which is expensive and provide environmental pollution. Therefore, legume inoculation with rhizobial inoculant is one way to ensure that specific rhizobial strain for that host plant is present in the soil at the proper time and in sufficient number to assure a quick and effective nodulation and efficient subsequent nitrogen fixation (Cleyet-Marel, 1988).

2.1 Rhizobial inoculant

2.1.1 History

Since 1886, Hellriegel and Wilfarth discover that bacteria caused the formation of the nitrogen-fixing nodules. Then, the isolation of rhizobia from the nodules as

pure cultures opened the way for artificial inoculation to replace the 'soil transfer' method, which dry soil, from a location where the legume had been grown previously, was coated onto the seed just before sowing (Date, 2001). This dust method was modified to the "soil-paste or muddy water process", in which the soil was mixed with water before pouring over the seed (Burlison et al., 1930). The first commercial pure (agar) culture inoculants have been patented by Nobbe and Hiltner in 1896. Their patented culture was placed on the market under the name Nitragin, which consist of a pure culture of desired strain of rhizobia grown in flat glass bottle containing only a small amount of solid gelatin medium. This material was either to be applied to seed or mixed with soil and spread over the field (Smith, 1992). Then, solid carrier such as soil or peat was first suggested in 1914 (Date, 2001). Present day inoculant production techniques have been changed from those of the early 1900s. Even many types of inoculant have been investigated, peat is the best carrier and is widely accepted in the inoculant industry. However, the challenge today is to develop further improved inoculant formulations and methods of application.

2.1.2 The need for inoculation

Although rhizobia seem to be widely distributed in the soil, however soil in different places contains different strain of rhizobia and these rhizobia may not effective for nitrogen fixation, and may not appropriate for legume need to plant. Some soil may have effective rhizobial strain, but the number of rhizobia is low or containing higher number of ineffective strain (Herridge et al., 2002). Inoculation of legume seed is a simple and practical means of ensuring effective nitrogen fixation. However, to answer the question "Is inoculation of seed necessary?", is critical, even

the using of rhizobial inoculant is not necessary in that area (over inoculating). Therefore, Allen (1958) has listed four indicators that, if positive, the inoculation would be beneficial:

- The absence of the same or symbiotically-related legume in the immediate past history of the land
- Poor nodulation when the same crop was grown on the land previously
- When the legume followed a non-legume in the rotation
- When the land was undergoing reclamation

However, the decision to inoculate is usually based on a result from experimental plots. Date (1977) has explained the experiments and trials referred to the necessary of using rhizobial inoculant. The experiments included uninoculated control plants as well as those inoculated with effective rhizobia and N fertilizer control treatments. The uninoculated treatment evaluates the presence or absence of indigenous strains and, if indigenous strains are present, provides some assessment of their symbiotic effectiveness compared with plants inoculated with selected strains of rhizobia. The inoculated treatment assesses the ability of a known effective strain of rhizobia to colonize the rhizosphere and to compete for nodule forming sites with any indigenous strain that may be present. The nitrogen treatment is included to ensure that the legume has the potential to grow well when provided with adequate nitrogen and that growth is not limited by other factors such as phosphorus deficiency or low soil moisture availability. The trials were listed in Table 2.1.

Table 2.1 Legume experiments for evaluation the necessary of rhizobial inoculant

Situation	Uninoculated		Inoculated		Nitrogen fertilizer	
	Nodulation[a]	Plant growth	Nodulation[a]	Plant growth	Nodulation [a,b]	Plant growth
1	-	Poor	-	Poor	-	Poor
2	-	Poor	-	Poor	-	Good
3	-	Poor	+E	Good	+ or -	Good
4	-	Poor	+I	Poor	+ or -	Good
5	+I	Poor	+E	Good	+ or -	Good
6	+I	Poor	+I	Poor	+ or -	Good
7	+E	Good	+E	Good	+ or -	Good

[a](-) no nodulation; (+) nodulation; E: effective in N-fixation; I: ineffective

[b]Plants may or may not be nodulated depending on whether the applied N inhibits nodule formation

The different results demonstrated in Table 2.1 could be explained in each situation as following:

1. No nodulation and poor plant growth of the uninoculated plants, indicating that suitable native rhizobia are not present in the soil. No nodules and lack of a growth response from either the inoculated or the nitrogen fertilizer treatments, indicating that some factor other than N, e.g. phosphorus supply, is limiting plant growth.

2. No nodulation and poor growth of uninoculated and inoculated plants but good growth with nitrogen fertilizer, indicating that either the inoculum strain or the quality of the inoculant supplied was unsatisfactory.

3. No nodulation and poor growth of the uninoculated plants, but effective nodulation of inoculated plants with growth similar to that in the nitrogen fertilizer treatment, indicating that the inoculant strain has been successful in forming nodules and fixing N_2.

4. No nodulation and poor growth of uninoculated plants. Inoculated plants are well nodulated though growth compared with the nitrogen fertilizer treatment is poor. This indicates that the inoculum strain was unsuited to the test legume.

5. Uninoculated plants grow poorly but have numerous ineffective nodules, indicating nodulation by an unsuitable native strain. Inoculated plants are effectively nodulated and grow well, demonstrating that the inoculum strain was both competitive with the native strains for nodule sites and effective in N_2 fixation with the test host.

6. Uninoculated and inoculated plants nodulated ineffectively and grow poorly in comparison with the N fertilizer treatment, suggesting either that native rhizobia were ineffective and competitive in nodule formation, or that the inoculum strain was ineffective in N_2 fixation. It would be necessary in this case to use some strain-identifying technique such as serology (Vincent, 1970), antibiotic resistance (Obaton, 1971) or molecular markers (Richardson et al., 1995) to determine the proportion of nodules formed by the inoculum strain.

7. Uninoculated plants with effective nodules and good growth compared with the other two treatments, indicating that native rhizobia suitable for the host legume

are present in the soil. With no response to inoculation in this situation, the nodules on the plants in the inoculated treatment would most likely be formed by the indigenous strains. Again, this could be checked by one of the strain identifying techniques. Under rare circumstances it is possible that the inoculated treatment in this situation could be nodulated and poorly grown, indicating a highly competitive but ineffective strain in the inoculated treatment. An alternative result could be no nodulation in any treatment but good growth in all treatments indicating a high background level of available soil nitrogen.

Therefore, there is a need for legume inoculation when the indigenous rhizobia were ineffective, and there is no high background level of available nitrogen in soil. Also inoculant should contain high numbers of effective rhizobia in order to out-compete populations of ineffective rhizobia in the soil or to build up populations where soil conditions have limited their persistence. Then, when rhizobial inoculant is decided to use, the inoculant types and inoculation techniques must be considered in which it will be appropriate for legume planting.

2.1.3 Types of inoculant

Rhizobial inoculant could be divided into many types depend on the form of inoculant.

2.1.3.1 Agar inoculant

This form is the first commercial inoculants (manufactured by the Nitragin Co., Milwaukee, USA.), based on the finding of Nobbe and Hilter (1896). It was produced on gelatin, and later on agar or nutrient media. One of the techniques

recommended for applying agar-based inoculant was application of suspension directly to the seed. This method proved so convenient and effectual that, with modifications, it is still in use for virtually all legume inoculation to now a day. The major drawback of agar inoculant is the high mortality rate during the drying phase immediately following application to the seed. This mortality can be reduced significantly by the addition of 9% (w/v) maltose to the suspending fluid (Brockwell, 1982).

2.1.3.2 Polymer-gel entrapped inoculant

Possibly, the most innovative technology for rhizobial inoculants in recent times involves the encapsulation of the bacteria in polymer microcapsules, beads or pellets. Polyacrylamide and alginate are the 2 most commonly tested gels. The inoculant is formulated by mixing a culture of the bacteria with a hardening or gelling compound (adjuvant) in a polymer solution. Bulking or nutritive additives such as bran or clay can be added and the mixture added drop-wise into a solution containing a calcium salt to solidify and form the gel pellets. Particle size and shape can be controlled by varying the drop-forming system. The particles are then dried for ease of packaging and handling. Hardness, moisture uptake or release and rate of degradation of the beads can be adjusted by varying the amount and grade of adjuvant and polymer used to prepare the pellets (Bashan, 1986; Walter and Paau, 1993). Particles of polyacrylamide-entrapped *Rhizobium* (PER) (Dommergues et al., 1979) resulted in an inoculant that compared very favorably with peat-based inoculant when kept at suitable moisture content. Rhizobia entrapped in a similar way with alginate (AER, alginate-entrapped *Rhizobium*) or a mixture of xanthan and carob gum (XER,

xantan-entrapped *Rhizobium*) made satisfactory inoculants and were not different to PER in the wet form after 50 days of storage at 28°C. Dried PER, AER and XER formulations were of poorer quality (number of cells per unit) and were not consistently better than peat either as soil or seed inoculant (Jung et al., 1982). Although polymer-entrapped rhizobia formulations can provide an alternative to powdered organic carrier-based inoculants, they have the disadvantage that the best results are obtained when they are maintained in their 'wet' condition. Drying caused significant loss of viability of the same order as in dried peat and on a volume basis had fewer viable cells (Date, 2001). Drying PER appeared to be harmful since nodulation and total N of aerial parts were markedly decreased when dried (Dommergues et al., 1979).

2.1.3.3 Lyophilized inoculant

Lyophilization is widely used for preservation of microorganisms, but the first appears to be an instance of commercial application was a freeze-dried inoculant produce by an Australian firm for a short period in 1958 (Brockwell, 1982). McLeod and Roughley (1961) found that lyophilized cultures could provide good nodulation in the field but this is likely to be restricted to favorable conditions, as survival of lyophilized rhizobia on seed is poor (Vincent, 1965). Kremer and Peterson (1982) have reported that peanut oil and soybean oil could overcome poor survival of rhizobia. Their claim was that freeze-dried rhizobia suspended in dried oil were protected, particularly against high soil temperatures.

2.1.3.4 Solid-based inoculant

Solid-based inoculants now form the vast majority of cultures market. A history of the development of the legume inoculants based on solid carriers, mainly soil and peat, but also other materials have been studied, such as soils, clays, coal, charcoal, perlite, rock phosphate, bagasse, filter mud, vermiculite, and ground plant residues (Brockwell, 1982; Stephens and Rask, 2000). However, the best researched and the most frequently used material for inoculant production is peat. A large number of studies have shown that peat provides better protection for rhizobia in the package and on inoculated seed than other materials due to peat rich in organic matter and mineral (Somasegaran and Hoben, 1994). For commercial purposes, a safe storage period of 6 months can be expected from peat-based inoculant (Roughley and Vincent, 1967). The physical and chemical analyses of well-known peats are showed in table 2.2 and 2.3.

Table 2.2 Characteristics of sedge peat used for commercial inoculant production in the United States

Sedge Peat Contents	Amounts
Total N (%)	1.62
Organic matter (%)	86.80
Ash (%)	13.20
Exchangeable K (ppm)	62.00
N as NH_4 and NO_3 (ppm)	94.00
Available P (ppm)	12.00
pH	4.5-5.0
Moisture (%)	7-8

Table 2.2 (continued)

	Analysis of ash (%)
K	1.12
P	0.33
Ca	5.21
Mg	1.14
Fe	2.10
Si	28.00
Al	6.32
Na	0.52

Modified from Burton (1979)

Table 2.3 Characteristics of Badenoch peat used for commercial inoculant production in Australia

Characteristics	Range	Mean
Organic matter (%)	28.8-75.4	64.3
Organic C (%)	16.4-42.1	36.1
Mineral matter (%)	10.0-16.0	12.1
Total soluble salts (%)	0.09-1.50	0.87
Cl (%)	0.01-0.31	0.11
N (%)	0.89-2.30	1.83
K_2O (%)	0.12-0.17	-
P_2O_5 (%)	0.09-0.22	-
Water-holding capacity (%)	216-522	320
C/N ratio	15.0-17.5	16.7
pH	5.8-7.8	6.8

Modified from Roughley (1970)

The type of peat affects the number of rhizobial cells which develop, and their subsequent survival during storage. Notably, the chemical analysis of the peat may not always confirm its quality as a suitable material. The acceptable peat can be made only on actual tests of its suitability for growth and survival of particular strains of rhizobia. Moreover, its suitability can also vary depending on source and batch in each production (Roughley and Vincent, 1967). The production of peat inoculants is complicated and need many steps, started by selection and screening to remove debris, such as stones, roots, and then drying with forced air. Drying temperature should not exceed 100°C as higher temperatures can degrade peat and release toxic substances, which can also restrict subsequent growth and survival of rhizobia. The peat is then ground in a high speed hammer mill and passes through a shifting machine. Peat with a particle size of 10-40 μm is collected for seed coating, and peat with particle size of 500-1500 μm is used for production of soil implant (granular form) inoculant. Most peats are too acidic to use as carrier without prior neutralization. Therefore, peat must be neutralized with precipitated $CaCO_3$ (pH 6.5-7.0). This neutralizing agent is usually added to the peat before milling (Somasegaran and Hoben, 1994). Both sterilized and non sterilized peats are used in commercial production systems. Peat can be sterilized by autoclaving at 15 lb/in^2 pressure, 121°C for at least 60 minutes. However, heat sterilization of some peat has been found to produce undesirable changes and to release toxins. Therefore, Gamma-irradiation sterilization is preferred. This sterilization cause sterile peat production is more costly than non-sterile peat. So, the non-sterilized peat is available in commercial legume inoculant. The inoculants made in this way have known to carry a considerable viable non-rhizobial contaminant but these contaminating organisms have been generally ignored. Three

basic contaminant types were observed, such as bacteria, actinomycetes, and fungi. These include the possibilities of pathogenecity to human, animal, plant or rhizobia, which reduce the effectiveness of inoculant (Olsen et al., 1996).

2.1.3.5 Liquid inoculant

According to complicated processing of solid-based inoculant, liquid inoculants are ideal because preparation and handling are simple and purity easily confirmed. In the past, commercial liquid inoculants have been marketed only sporadically, basically because of the difficulties which arise in maintaining biological control after the cultures leave the manufacturer (Brockwell, 1982). Manufacturers seem to have overcome the problem of deterioration by concentrating the broth inoculant with centrifugation, placing it in plastic containers, freezing it, and transporting it to the user in a frozen state packed in dry ice. Then, the concentrated rhizobia are diluted with water before use. Nevertheless, the shelf life and the need of cool temperature storage are limited the use of liquid inoculant (Stephens and Rask, 2000). However, the liquid inoculants currently available display various broth formulations, each the result of individual producer's research and development activities. Brockwell and Bottomley (1995) has been reported the liquid inoculant which packaged in dispenser bottles, and can be used as a seed inoculant or for delivery directly into seed bed. This form of liquid inoculant has good storage characteristics in the bottle or on the seed, adheres tenaciously to the seed coat without the need for adhesive, and gives rise to nodulation and N_2 fixation as good as can be obtained with peat inoculant. Moreover, several compounds have been studied for their protective function and added to liquid inoculant for promoting the survival of

rhizobia after inoculation. For example, polyvinylpyrrolidone (PVP) is the polymer that may bind toxic compounds release from seed coat, and also has high water binding capacity, which appears to slow drying of the inoculant after application to seed. FeEDTA and glycerol are added to supplement iron and carbon source to the rhizobia, glycerol can also protect cells from the effects of desiccation (Singleton et al., 2002). Therefore, liquid inoculation formulation with other additives can be moved toward for their commercialization instead of peat-based inoculant, which true peat are rare and can be exhausted in the future.

2.1.4 Inoculation techniques

Rhizobia may be introduced to legumes by inoculation of the seed or soil. Seed may be inoculated by farmers immediately prior to sowing or custom inoculated by local seed merchants with coating facilities to be sown within a week. However, legume seed may be commercially inoculated and stored prior to its sale. This product is commonly referred to as pre-inoculated seed. Alternative methods to seed coating include direct inoculation of the soil using peat inoculants suspended in water or inoculants formulated as liquids or granules. Both seed and soil inoculation techniques have advantages and disadvantages. Selection of a method depends on the availability of equipment, seed size and fragility of cotyledons, the presence of seed applied fungicides and convenience. The fundamental practices and appraisal of methods for seed and seed bed inoculation and of preinoculation are concluded in table 2.4.

Table 2.4 Methods of legume inoculation and their appraisal

Methods	Procedure	Appraisal
Dusting	Dry, peat-based inoculant poured on to seed in seed-box.	- Quick, simple. - Much of the inoculant is shed before the seed reach the soil. - No longer recommended.
Slurry	Peat-based inoculant suspended in water or an adhesive solution, applied to seed, mixed together, and allowed to dry before sowing.	- Well understood and no special equipment required. - Necessary to dry seed away from direct sunlight. - Some splitting of seed may occur during handling. - Slow and labour-consumptive, especially where sowing rates are heavy. - Reinoculation necessary if not sown within 48 h. - Not suitable when fungicidal or insecticidal seed dressings have been used.
Solid inoculant	Polythene beads, marble chips or other freely-flowing core material coated with peat-based	- Gives consistently excellent nodulation. - Maintains viability well. - Suitable for inoculation of

Solid inoculant (continued)	inoculant and applied in the row with the seed. Peat-based inoculant extruded into freely-flowing pellets and applied in the row with the seed.	pesticide-treated seed. - Available commercially in U.S.A. but not in other countries. - Complicated to prepare on the farm. - Drills fitted with special (insecticide) hoppers necessary for application.
Seed pelleting	Peat-based inoculant suspended in adhesive, applied to seed, mixed together, then the finely ground coating material is added to sticky seed and mixed (preferably by rolling) until each seed is evenly coated, dry and discrete.	- Prevents shedding of inoculant. - Protects inoculants against deleterious soil environmental factors, e. g. acidity. - Well adapted to surface seeding. - Time consuming preparation. - Coating material may slough off and cause blockages in seeding machinery. - High inoculant mortality rate observed in many commercially prepared pelleted seed samples.
Spray inoculation	Peat-based inoculant suspended in water or liquid inoculant sprayed into seed bed alongside	- Gives consistently excellent nodulation. - Suitable for inoculation of pesticide-treated seed (in case

Spray inoculation (continued)	seed. (liquid inoculant may also be applied to seed)	spray inoculant into seed bed). - No complicated preparation procedure.
Granular inoculation	Prepared from peat, by impregnating pre-granulated peat, in a manner similar to that for preparing normal peat cultures, particle sized (0.2–0.8 mm), and sown with seed in seedbed.	- Suitable for inoculation of pesticide-treated seed. - Granules may absorb more moisture and caused the greatest reduction in number of viable rhizobia.
Preinoculation	Inoculation of legume seed with rhizobia prior to sale and usually implying a period of storage of the inoculated seed before sowing.	- Very convenient for farmers. - Mortality rate of inoculant often very high resulting in low levels of inoculation at time of sowing.

Modified from Brockwell (1982), Date (2001), and Deaker et al. (2004)

2.1.5 Production of legume inoculant

The three concerns in inoculant production are the quality and processing of the carrier, the purity and efficiency in nodulation and N_2 fixation, of the culture, and achieving adequate cell numbers in broth culture and finished product. Therefore, to

achieve this goal, there are four important steps of inoculant production must be considered.

2.1.5.1 Strain selection

To optimize nitrogen fixation in commercially important legumes, culture organisms need to be competitive with the indigenous populations and be efficient in N_2 fixation upon nodule formation. There is a need to select strains for specific geographic, soil and environmental regions and, ideally, for individual varieties of a given legume species. The top performing strain(s) for a given market area must be compatible with the commonly grown crop cultivars in that area, and display the adaptability to be effective and efficient over a range of geographic and environmental conditions. This adaptability is an obvious requirement since soil types, moisture patterns and cultivar preferences can alter over small geographic areas. Lynch (1983), stated "Each microbial species will have an optimum for each physical or chemical factor and its growth, or activity, will decline either side of that optimum, governing its contribution to the total population." Further it has been noted that microbial preferences can change within macro and micro climates determined by soil texture (Mahler and Wollum, 1981; Osa-Afiana and Alexander, 1982), structure, aeration, pH (Cassman et al., 1981), temperature and available moisture (Hardarson and Jones, 1979; Clark et al., 1986; Sprent et al., 1986), nutrient status, organic matter content (Semu and Hume, 1979), and weather. It is apparent, therefore, that rhizobial activity can vary greatly with environmental pressures, with clear differences in response found both between and within species. Selection of an appropriate organism is critical and cannot be a process carried out over a single year of field trials

and over few locations. There have been reported that the most effective strains for a given crop, in a given geographic region, were often strains isolated from that region (Shishido and Pepper, 1990; Sattar et al., 1995). Competitiveness of selected strain(s) with indigenous populations of rhizobia is also a major factor in strain selection (Winaro and Lie, 1979; Broughton et al., 1982; van Rensburg and Strijdom, 1982; Fesenko et al., 1995). Therefore, the means of recovering and typing nodule occupancy are definite need for any organization developing inoculants. To merely use a strain displaying acceptable N_2 fixation *in-vitro* is not acceptable. The inoculant organism must display both acceptable N_2 fixation and competitiveness, for the inoculant to be effective.

2.1.5.2 Culture production

Media used in the cultivation of rhizobia must contain all nutritional requirements in a form suitable for rapid cell production. There is a need to ensure that the medium can support populations at, or exceeding, 10^9 c.f.u./ml. This value is being targeted for the production of sterile peat based products since the mother culture is diluted, with sterile deionized or reverse osmosis, water, to at least 1/50 and in some instances 1/250 prior to injection into the sterile peat. There are many formulations medium for rhizobia production (Table 2.5). Moreover, by-products from food processing, cheese whey, corn steep liquor, malt sprouts and malt bagasse have been reported to be acceptable carbon and nitrogen sources for rhizobial culture medium (Meade et al., 1985; Bissonnette et al., 1986; Balatti et al., 1991). However, the use of by-products from the food processing industry can be problematic since few such “waste” products can assure continuity of quality. Even the use of conventional

medium substrates, Skinner et al. (1977) noted that excessive use of yeast extract as a carbon and nitrogen source resulted in both cell morphological changes and decreased nodulation potential.

Table 2.5 Media composition for rhizobia production

Source: Norris and Date (1967)	
Mannitol	10.0 g
Yeast extract	1.0 g
K_2HPO_4	0.5 g
$MgSO_4.7H_2O$	0.8 g
NaCl	0.2 g
$FeCl_3.6H_2O$	0.01 g
Distilled water	1000 ml
Source: Burton (1967)	
Sucrose	10.0 g
Mannitol	2.0 g
K_2HPO_4	0.5 g
$MgSO_4.7H_2O$	0.2 g
NaCl	0.1 g
$CaCO_3$	0.25 g
Yeast autolysate	1.0 g
Distilled water	1000 ml

Table 2.5 (continued)

Source: Fred et al. (1932)	
Sucrose	10.0 g
Yeast extract	3.0 g
K_2HPO_4	0.75 g
$MgSO_4.7H_2O$	0.4 g
$CaCO_3$	0.4 g
Distilled water	1000 ml
Source: Arias and Martinez-Drets (1976)	
Glycerol	10.0 g
Yeast extract	3.0 g
KH_2PO_4	1.0 g
K_2HPO_4	1.0 g
$MgSO_4$	0.18 g
NaCl	0.2 g
$CaCl_2$	0.13 g
$FeCl_3.6H_2O$	0.004 g
NH_4Cl	0.5 g
Distilled water	1000 ml
Modified from Smith (1987)	

Rhizobia can be grown in the equipment of varying size and design depending on the production needs, available resources, and funding. It can be used since simplest of containers as bottles, round-bottomed flasks, shake flasks, or any of

a wide variety of fermenters (Smith, 1987). However, fermentation variables such as dissolved oxygen (DO), temperature, pH and impeller tip speed must be established for each organism. Current fermenter technology can provide continuous monitoring, and control of virtually all measurable parameters and as such custom production of mother cultures of any chosen *Rhizobium/Bradyrhizobium* species or strain is possible and, indeed, desirable. Commonly used DO values are between 90 and 100%, and temperature values between 25 and 28°C are the standard. Fermenter starter cultures are normally produced in swirl flasks, where culture pH and DO are rarely controlled. DO values of the starter cultures in swirl or stir flasks, have been reported to be about the 25±30% saturation range for mature cultures (Stephens and Rask, 2000). Lack of control of these variables in starter cultures can result in significant lag phase on transfer of the mother culture to the fermenter. This results in decreased population densities at the time of scheduled harvest. Moreover, the contamination of other microorganisms should be considered.

2.1.5.3 Carrier

Rhizobia are usually incorporated into solid carrier which serves several functions during the period from manufacturing through product use. The carrier becomes the major portion of the product and provides a convenient base to facilitate product packaging and final product application and use (Smith, 1987). Smith (1992) has noted that the raw materials chosen for use in conventional inoculants are determined by availability, consistency of quality and cost. The carrier must display two fundamental properties; it must support growth of the target organism and maintain desired populations of inoculant strains over an acceptable

time period. To achieve these goals a carrier must also display high water holding capacity and retention characteristics, display chemical and physical uniformity and be non-toxic to inoculant strains and environmentally safe. Carrier should easy to be sterilized by autoclaving or gamma irradiating, also it should readily available and inexpensive, has pH buffering capacity, sufficiently adhesive for effective application to seed, and has the property of cation-anion exchange capacities. It needs also to permit growth after introduction of the rhizobia, have an acceptable pH, rapidly release organisms upon use and be in abundant supply (Keyser et al., 1992). Peats fulfill the bulk of these requirements however the supply of this peat is becoming the important problem in many countries. Even many materials have been used as carrier, often the ability of an alternative carrier, or entrapment methodology, to sustain organism growth is marginal and also cost, consistency of supply and quality against adoption of these alternative materials.

2.1.5.4 Finished product and inoculant shelf life

Production considerations must be scheduled to suit employee work periods because a completely integrated system must be developed. This must be amenable to specified dates of culture harvest at which the culture always conforms to a set of measurable parameters (Stephens and Rask, 2000). Then after finish packaging, the finished product must be stored in the appropriate storage room. In temperate climates ambient temperatures and humidity may be utilized; however, air conditioning is advisable in warm weather, and refrigerated storage is ideal. Inoculants are perishable product which should not be submitted to conditions that enhance drying, such as increase temperatures and direct sunlight (Smith, 1987).

Shelf-life is an important inoculant characteristic for farmers and manufacturers. It has been shown many years ago that contaminants have detrimental effect on shelf-life of rhizobia (Date and Roughley, 1977). Therefore, the current trend is to consider that contaminants-free inoculants are preferred **(**Brockwell and Bottomley, 1995). In order to study the survival of *B. japonicum* in manufactured inoculants, Catroux et al. (1996) evaluated pure industrial products, stored up to 8 years, at 20°C. Peat and liquid *B. japonicum* inoculants can provide 10^9 viable *B. japonicum* per g or per ml (determined by plate counts) after 4 – 6 years storage. These data demonstrated that high standards of inoculant quality could be reached when using a sterilized carrier and pure cultures.

However, the discrepancies between plate counts and field efficacy have been observed. The evolution of *B. japonicum* physiological characteristics during long term storage may contribute to this discrepancy. Catroux et al. (1996) found that when the length of storage increased, the time for colony appearance on a medium without yeast extract and the time for nodule appearance in tube culture increased, indicating a decrease in the fitness of the surviving bacteria. Over this time, the bacteria were more sensitive to desiccation when inoculated onto soybean seeds. They have also tried in a field experiment to compare the relative percentage of efficient *B. japonicum* in stored inoculants with the viable cell number on plate counts. The number of efficient *B. japonicum* cells decreases over the time of storage (Table 2.6). These data underlined the need to have good quality control methods and good management in order to guarantee the efficacy of the products sold to the customer.

Quality assurance ensures that, at each stage of the production process, the product which did not meet defined criteria must be aborted. There are many

Table 2.6 Estimated percentage of efficient *B. japonicum* in stored peat inoculants

Storage (Year)	Inoculant (log c.f.u./g)	% efficient *B. japonicum*
1	9.59	36.10 ab
4	9.10	41.66 a
6	8.30	11.16 ab
7	7.26	3.42 b
8	7.31	5.41 b

Remark: Values in the same column followed by the same letter are not statistically different at $p = 0.05$ (modified from Catroux et al. (1996))

methods have been reported to use for inoculant quality evaluation, such as microscopic examination, plate count of viable cells, and most probable number (MPN) plant infection assay. Also the immunological techniques, such as cell agglutination reaction for rhizobial identity, immuno-spot blot and colony-lift immunoblot tests for rhizobial identity, and indirect fluorescent antibody identification of rhizobia in broth (Lupwayi et al., 2000). Regulation of inoculant quality is required, and the quality standards should be established. Regulation of inoculant quality standards varies from country to country and no set of international standards exist. Most inoculant standards establish a minimum number of viable rhizobial cells of the appropriate species or strain per unit weight of inoculant. At least 10^9 rhizobial cells/g are required in France (Catroux, 1991), Australia (Thompson, 1991), Rwanda (Scaglia, 1991), Zimbabwe and Kenya (Marufu et al., 1995); 10^8 cells/g in New Zealand and South Africa (Smith, 1992) and 5×10^7 cells/g in Thailand (Boonkerd, 1991). Granular products applied as soil inoculant are required to deliver at least 10^{11} cells/ha in Canada (Olsen et al., 1994).

However, the use of good quality inoculant product in some cases may not ensure the nodulation and N_2 fixation due to there might have other factors affect the survival and reduce the number of rhizobia on the legume seed before root hair infection. Therefore, it is necessary to know about some of these adverse factors for finally improving the highest efficiency of rhizobial inoculant.

2.2 Factors affecting survival of rhizobia on legume seed

Many environmental factors have been reported to have an effect on the rhizobia-legume symbiosis and nitrogen fixation, and also the persistence of rhizobia in the soil. These environmental factors are salt and osmotic stresses, soil moisture deficiency, high temperature and heat stress, soil acidity and alkalinity, and nutrient deficiency stress etc (Zahran, 1999; Hungria and Vargas, 2000). However, the first step of successful seed inoculation with rhizobial inoculant, the number of viable rhizobial cell on seed should be high enough to colonize the root hair and start their interaction between rhizobia and legume after seed germination. Since the death of rhizobia is common to all known inoculation procedures, especially seed inoculation technique. Applying higher quantities of inoculant is uneconomical and technically difficult. Alternatively, higher numbers of viable rhizobia per seed may be achieved by improving survival during seed inoculation. Therefore, the factors affecting survival of rhizobia on seed should be concerned.

2.2.1 Desiccation

Desiccation is a major contributing factor to poor survival on seed (Vincent et al., 1962). A study of the survival of *R. leguminosarum* bv. *trifolii* on glass beads

under dry conditions indicated that there were two distinct phases of death (Vincent et al., 1962). After an initial rapid decline in cell numbers between 0 and 24 h that coincided with a rapid loss of water, there followed a period during which water loss and death rate declined. Cells survived best at 100% relative humidity and no viable cells were detected after 27 h at relative humidities below 60%. Some research has demonstrated a direct relationship between water loss in peat and survival of rhizobia (Vincent, 1958). Further research indicated that tolerance to desiccation varies between strains of rhizobia. Bushby and Marshall (1977) found that slow-growing strains of rhizobia survived better than fast-growing strains after desiccation in sandy soil. In their experiments, air-dried soil (10 g) was inoculated with 4 ml of either fast- or slow-growing rhizobia to provide 10^6 cells/g of soil. After drying in a 28°C forced-draught oven overnight, only 10^2 cells of fast-growing rhizobia survived compared with 10^5 cells of slow-growing rhizobia /g of soil.

The stress of desiccation is further complicated by the toxic nature of O_2. Two stages of desiccation were identified; partial dehydration when the relative humidities were still high followed by dehydration where relative humidities are below 70% and where O_2 becomes toxic (Deaker et al., 2004). The targets of O_2 damage are proteins, membranes and nucleic acids (Potts, 1994). Protein damage through metal-catalyzed (Fe^{3+}) oxidation reactions and lipid peroxidation leads to a loss of membrane semi-permeability and ultimately cell lysis. Accumulation of breaks in the DNA molecule occurred during exposure of bacterial cells to the superoxide anion radical (O_2^-) and hydrogen peroxide (H_2O_2). O_2 has a detrimental effect on the survival of freeze-dried bacteria (Williams, 1954; Mellor, 1978). As a result freeze-dried cultures survive better stored under vacuum or N_2 than when stored in air (Mellor, 1978).

2.2.2 Temperature

Temperature starts to have an effect on survival of rhizobia on seed, since the inoculated seeds were stored in storage room before distribution to farmer. Low temperature (5°C) showed benefits survival of rhizobia, but is not practical (Date, 1968). Vincent (1958) demonstrated death rates of clover rhizobia on glass beads increased when storage temperatures were raised from 5 to 26 and 37°C. Bowen and Kennedy (1959) demonstrated the susceptibility of many rhizobia to temperatures of 40°C. Greatest stress from temperature is experienced when sowing onto the surface of dry soil. This is of particular concern in tropical areas where the soil temperatures regularly exceed 40°C at 5 cm and 50°C at 1 cm depth (Eaglesham and Ayanaba, 1984; Hafeez et al., 1991; Lal, 1993). While surface temperatures reach maxima of 70°C in soils around Roma, Queensland (McInnes and Date, 1999), and 62°C in sandy soils of Western Australia (Chatel and Parker, 1973).

The effects on survival of desiccation and temperature are not mutually exclusive. Rhizobia are more sensitive to moist heat than dry heat (Vincent et al., 1962; Wilkins, 1967). Wilkins (1967) found that nodule bacteria for *Medicago* spp. were capable of surviving for 32 h in dry soil at 60°C but did not survive for 5 h in moist soil at 55°C. Lyophilized rhizobia survive better at high temperatures than peat cultures (Kremer and Peterson, 1982, 1983; McInnes and Date, 1999) but the cells must be kept dry to maintain tolerance to high temperatures, which is difficult for practical. Moreover, Boonkerd et al. (2001) found low temperature also affect the survival of rhizobia. They found that the population of rhizobia decreases due to dry, low temperature, and lack of humidity in soil during winter time in Thailand. The *B.*

japonicum which mainly nodulate soybean that could be found in rainy season was disappeared in winter.

2.2.3 Acidity

Soil acidity effects on legume nodulation include reduced survival and growth of rhizobia in soil and seed. Acidity has often been implicated in legume and inoculation failures. Rhizobial inoculant with limestone pelletting seed has been used for protection rhizobia on seed from the toxicity effects of acid soil (Loneragan et al., 1955). Acid tolerant rhizobial strains tend to benefit for legume inoculant production in order to survive and successful nodulation in acid condition. Acidity normally has much effect on fast-growing rhizobia than slow-growing rhizobia (Sadowski and Graham, 1998). However, there are some strains of fast-growing rhizobia are highly acid tolerance. The acid-tolerant strains of *R. loti* demonstrate a comparative advantage over acid-sensitive strains in the ability to nodulate their host legume at pH 4.5. *R. tropici* and *R. loti* are moderately acid tolerant (Wood et al., 1988), while *R. meliloti* is very sensitive to acid stress (Brockwell et al., 1991; Tiwari et al., 1992). However, *R. meliloti* WSM 419 has recently been shown (Clarke et al., 1993) to perform satisfactorily in the field in acidic soils (pH 5.0 to 5.5)

As pH outside the cell starts to become more acidic, internal pH homeostasis mechanisms are able to maintain pH inside the cell (pH 7.6-7.8 for the *Enterobacteriaceae*). This will create a large ΔpH, because the interior pH of the cell remains relatively neutral. As the external pH declines further, pH homeostasis mechanisms eventually fail, proton leakage increases, and internal pH acidifies with

ensuring damage to internal macromolecues and acid-sensitive metabolites (Hall et al., 1995).

Acid condition can be occurred at rhizosphere of plant, since the root of legumes are known to secrete H^+ and organic acids that sharply decrease the pH of the rhizosphere up to 2 units below that of the surrounding bulk soil (Marschner, 1995). This condition also affects rhizobia in rhizosphere. Moreover, soil acidity is often associated with increased aluminium toxicity, because aluminium is more soluble when soil pH was low. The total soluble of aluminium in the soil solution can be more than 1,000 μM in acid sulphate soil (Kamprath, 1978). The total concentration of monomeric aluminium in acid soils is the sum of concentrations of various monomeric species, such as Al^{3+}, $Al(OH)^{2+}$, $Al(OH)_2^+$, and $Al(OH)_3$. The monomeric hydrolysis of aluminium is significant at pH 4.0, and more than 80% of total soluble aluminium is hydrolyzed by pH 4.9 (Kinraide, 1991). Aluminium toxicity affects the growth of rhizobia (Cooper et al., 1983; Coventry and Evans, 1989; Campo, 1995), and symbiosis (Murphy et al., 1984; Brady et al., 1990; Campo, 1995). The nature and site of action of aluminium ions remain undefined. Studies by Johnson and Wood (1987), Matsumoto (1991) and Wood (1995) have indicated that Al ions act by binding to DNA, interfering with cell division. However, since both aluminium-sensitive and aluminium-tolerant strains bound similar amounts of aluminium, a DNA repair mechanism must exist in tolerant strains. However, Richardson et al. (1988) found that 7.5 mM aluminium depressed *nod* gene expression at low pH (4.8). Aluminium also toxic to plant by inhibition the root growth results in a reduced and damaged root system. The rapidity of this response indicates that aluminium first

inhibits root cell expansion and elongation; however, over the longer term, cell division is also inhibited (Kochian et al., 2004).

2.2.4 Toxin from seed exudates

Water-soluble substances from the seed coat may be inhibitory to rhizobia. There have been noted that more clover rhizobia died on seeds than on glass beads, possibly due to inhibitory seed coat diffusates (Vincent, 1958). Vincent et al. (1962) repeated these experiments and found that the drying stage (0–27 h) could be sub-divided into two stages, 0–5 h and 5–27 h. Higher death rates in the first 5 h suggested that inhibitory substances were most active when the seed contained readily available moisture. Exudates from legume seeds of different genera and cultivars of arrowleaf clover varied greatly in toxicity (Materon and Weaver, 1984a,b). Bowen (1961) demonstrated high toxicity seed exudates from *Centrosema pubescens* and low toxicity of those from *Medicago sativa* cv. *Hunter River*. Thompson (1960) found that toxicity of water extracts of clover seeds was higher after extraction for 6–8 h than 24 h. It was speculated that the disappearance of the inhibitory substances over time may be due to enzymic inactivation or masking by non-inhibitory substances produced in the later hours. Thompson (1960), Bowen (1961) and Masterson (1962) agreed that soybean seed coat toxins were not altered by autoclaving.

The active compounds were analogous to polyphenolic compounds with the tri-hydroxilic grouping on the phenolic B-ring such as delphinidin, quercetin, myricetin and tannins (Fottrell et al., 1964; Thompson, 1964; Masterson, 1965). Ali and Loynachan (1990) investigated the toxic compounds in soybean seed varieties, Corsoy 79 and BSR 101. The toxins were water soluble, thermostable and had

molecular size less than 2,000 Daltons. Genistein and daidzein were detected in high amount from soybean seed-exudates by using HPLC (Kape et al., 1992). Both genistein and daidzein are isoflavonoids, which were classified in phytoalexin (D'Arcy Lameta, 1985). Young and Paterson (1980) found seeds of *M. sativa* contained cyanidin, which was not toxic to rhizobia, and suggested that selection of non-toxic genotypes could be useful to assist survival during seed inoculation. Materon and Weaver (1984a) tested this hypothesis using non-toxic seeds of arrowleaf clover and results supported that the nontoxic seeds maintained a larger rhizobial population than a toxic variety.

2.3 Development of rhizobial liquid inoculant production strategies

According to problems associated with rhizobial liquid inoculant production, and effect of environmental factors that reduce survival of inoculated rhizobial cell (chapter 1), there were some strategies used for improving the production and survival of rhizobial inoculant in this study. Since inoculant composed of three main components; therefore, each component could be improved as following.

2.3.1 Culture medium

Numerous investigations have examined the nutritional diversity of carbon utilization by rhizobia, and they could be divided into two groups when carbon utilization was used as criterion. Fast-growing rhizobia are capable of growing on variety of carbon substrates, whereas slow-growing rhizobia are more limited in their ability to use diverse carbon sources. Fast-growing rhizobia are able to use a broad range of hexoses, pentoses, disaccharides, trisaccharides, and organic acids. On the

other hand, slow-growing rhizobia are unable to use disaccharides, trisaccharides, and organic acids for growth (Stowers, 1985). Mannitol is the traditional carbon source for all rhizobia. However, mannitol is cost of commercial mass culture. Sucrose is normally use for fast-grower rhizobia commercial mass culture production (Smith, 1987). Moreover, agricultural wastes, such as malt extract, whey, and molasses have been reported to use for alternative ingredients for development of the inoculant industry (Gulati, 1979).

It has been known that raw starch material, which has low cost, can be changed to be sugar by using biotechnological process (Chumkhunthod et al., 2001). Starch is composed of two polymers, α-D-glucose amylose and amylopectin in the form of linear and branch structure, respectively. Some groups of bacteria and fungi are able to produce α-amylase, β-amylase or amyloglucosidase (Zeikus and Johnson, 1991), these enzymes change starch to be maltose and glucose that can be utilized by fast-growing rhizobia. However, slow growing bradyrhizobia cannot use sugars produced from starch (Martinez-de Drets and Arias, 1972). However, glycerol has been reported to be one of the most universally used carbon sources among rhizobia, and glycerol can be produced from yeast fermentation process (Radler and Schutz, 1982; Pandey and Parekh, 1985; Vijaikishore and Karanth, 1987; Romano et al., 1997). Therefore, sugars produced from low cost starch material can be used directly in yeast fermentation process in order to produce glycerol as a sole of carbon source, and yeast cell derived from fermentation could be supplied as a nutrient source for slow growing bradyrhizobia cultivation.

Normally, glycerol was formed in high amount, when higher sugar concentration was used for fermentation (Radler and Schutz, 1982). However, it

could sometime retard the fermentation rate (Vijaikishore and Karanth, 1987). Moreover, it has effected to the cost of production. Therefore, the fermentation of yeast under special conditions that can improve glycerol production from low concentration of glucose is interesting.

There have been reported three kinds of glucose metabolism in yeast under anaerobic condition (James, 1928). The first type is under normal condition (slightly acid), *S. cerevisiae* uses the EMP pathway for glucose metabolism. The major products from this reaction are carbon dioxide and ethanol (Moat et al., 2002). This reaction also calls Gay-Lussac reaction. However, the reaction of fermentation changes when sodium sulfite is added into medium. In this second type reaction, glycerol is produced as a major product, and acetaldehyde is trapped and form bisulfite addition complex. Then, ethanol cannot be produced from acetaldehyde. The third reaction is occurred under alkaline condition (suitable at pH 7.0-8.8). The reaction will be altered in which one mole of acetic acid is produced for each two moles of glycerol.

$C_6H_{12}O_6 \longrightarrow 2C_2H_5OH + 2CO_2$ (1^{st} type reaction)

$C_6H_{12}O_6 + HSO_3^- \longrightarrow C_3H_8O_3 + CH_3CHO\text{-}HSO_3^- + CO_2$ (2^{nd} type reaction)

$2C_6H_{12}O_6 + H_2O \longrightarrow 2C_3H_8O_3 + C_2H_5OH + CH_3COOH + 2CO_2$ (3^{rd} type reaction)

However, the strain of yeast has directly effected to glycerol production even in the condition that promote glycerol production because the amount of enzyme glycerol-3-phosphate dehydrogenase presence in each strain is different. Therefore, the capacity to form glycerol during fermentation in each strain of yeast is also

different (Radler and Schutz, 1982). In this work, the production of glycerol under alkaline condition was selected for study, because adding sodium sulfite may cause toxic to bradyrhizobia. Therefore, the alkaline-reacting salt, $CaCO_3$ was added to maintain alkaline condition (Lie et al., 1992).

The processes of conversion low cost starch material to sugar and glycerol, and also nutrients from yeast cell derived from fermentation would be the beneficial method for producing rhizobial culture medium in order to reduce the cost of liquid inoculant production.

2.3.2 Additives

There are many kinds of additives have been used for improving rhizobial survival.

2.3.2.1 Sugars, amino acids and sugar alcohols

In early studies on the freeze-drying of bacteria, the nature of the suspending media was identified as an important aid to survival (Heller, 1941; Annear, 1956, 1962; Vincent, 1958). Extensive research has been carried out on the use of bacterial nutrients as suspending agents for freeze-drying and storage of cells (Heller, 1941; Appleman and Sears, 1944; Annear, 1956, 1962; Redway and Lapage, 1974; Dye, 1982). Heller (1941) investigated the protective effects of crystalline compounds and colloids during desiccation of *Streptococcus pyogenes* C203 and *Escherichia coli* (Deaker et al., 2004). Sucrose proved to be a superior suspending agent to glucose, xylose, tryptophane, salicin, saline and water for both species. Heller concluded that survival was related to the assimilability and solubility of the compound. Furthermore,

Vincent (1958) reported 24–44% of cells suspended in a 10% sucrose solution survived primarily drying whereas only 0.1% survived when suspended in water. Sucrose was a superior suspending agent to sorbitol, mannitol, lysine, amino acid mixtures, milk and yeast mannitol broth. The poor survival of rhizobia on seeds and beads was improved by the addition of sucrose. McLeod and Roughley (1961) found that the incorporation of 10% sucrose into yeast mannitol broth improved the survival on glass beads compared with unamended broth. Elsewhere, Vincent et al. (1962) reported survival of broth cultures of *R. leguminosarum* bv. *trifolii* on glass beads was much higher after suspension in a solution of maltose than when suspended in other sugars, sorbitol and sodium chloride.

To account for the maintenance of biological integrity during desiccation, a 'water replacement hypothesis' was developed by Crowe and Crowe (1986). Some desiccation-tolerant cells accumulate large amounts of the disaccharides trehalose and sucrose (Crowe et al., 2001). In systems where carbohydrates are dried in the presence of proteins, carbohydrates lose the capacity to form intermolecular hydrogen bonds. Instead, hydrogen bonding occurs between the carbohydrate and protein preserving its structure (Tsevtkov et al., 1989). Similarly, hydrogen bonding occurs between trehalose and phospholipids in membranes. Some models suggest a mechanism whereby trehalose fits between phosphates of adjacent phospholipids replacing water molecules (Gaber et al., 1986). Recent models indicate that the mechanism is simply related to the ability of the solute to physically decrease the force exerted on membranes as they approach one another under desiccation, decreasing membrane fluid-to-gel phase transitions (Bryant et al., 2001). Several applications for trehalose have been reported including the stabilization of vaccines

and liposomes (Crowe et al., 2001). It is produced in response to stress by yeasts (Mansure et al., 1997). However, for trehalose to stabilize cells it must be on either side of the membrane (Crowe et al., 1998). Trehalose may accumulate by transport from the environment or through *de novo* synthesis. Streeter (1985) found all species of *Rhizobium* accumulated trehalose to varying degrees and in most slow-growing strains, trehalose accounted for greater than 80% of the total mono- and disaccharides. There was an apparent relationship between the high accumulation of trehalose and survival in water culture. *B. japonicum* survived better in water culture than the fast-growing strains. None of the slow-growing strains tested grew with trehalose as the sole C source indicating that these strains may be deficient in trehalose-uptake systems and that accumulation is by *de novo* synthesis, while fast-growing rhizobia could uptake trehalose as carbon source (Stowers, 1985), suggested that adding more trehalose in medium may improve survival of fast-growing rhizobia. A membrane-transport protein for trehalose was identified in yeast (*S. cerevisiae*) and *Thermococcus litoralis* (Eleutherio et al., 1993; Han et al., 1995; Diez et al., 2001). Crowe et al. (2001) suggested that incorporation of the genes regulating its expression into cells without a transport protein might be a useful way of introducing trehalose into cells. An increase in the trehalose content of rhizobial cells may result in improved desiccation tolerance. However, addition of sugars could not negate the effects of inhibitory substances from the seed coat.

2.3.2.2 Polymers

Certain properties of polymers can be identified as having a beneficial effect on survival. Polymers should logically be non-toxic and free from preservatives

that may be harmful to bacteria. A complex chemical nature would be advantageous in order to faster-growing antagonists in the soil could not rapidly utilize the polymeric coating and out-compete the rhizobia. The polymer should also be dispersible in water to allow release of rhizobia from the polymer matrix upon wetting and their subsequent multiplication in the rhizosphere. Not only water dispersible of polymer, but the nature of water within the polymer matrix is critical to survival. Availability of water to act as a solvent is of great importance in biological systems (Mugnier and Jung, 1985).

The importance of colloidal stabilization on the survival of dried bacteria is also interesting. Heller (1941) demonstrated that the protective colloid properties of the suspending agent were positively correlated to survival. Steric stabilization is thought to be widespread in biological systems due to the consistent occurrence of cells bathed in solutions of high ionic strength and the abundance of water-soluble polymers. Colloids may be stabilized by several mechanisms: electrostatic, steric, depletion or electrosteric (Napper, 1983). Steric stabilization occurs when amphipathic polymers, having hydrophobic and hydrophilic parts, are attached to the surfaces of the colloidal particles. If the particles to be stabilized are hydrophobic in aqueous solution, then the hydrophobic part of the polymer binds to the particles and the hydrophilic ends repel one another thus maintaining the particles in suspension. One of the most important stabilizers in aqueous dispersions is polyvinyl alcohol (PVA). This is prepared by the hydrolysis of polyvinyl acetate (PVAc). As concentrations of salt increase in the cell environment under desiccation stress, stabilizing polymers may be useful in reducing the extent of protein precipitation or the coagulation of cells. Maintenance of macromolecular structure

may improve biological integrity thus leading to improved survival (Deaker et al., 2004).

Considering in the interaction with some seed exposes rhizobia to inhibitory substances from the seed coat. These substances have been identified as polyphenols and their inhibitory effect can be inactivated by the addition of materials capable of phenolic adsorption. Gum arabic is a complex carbohydrate extracted from *Acacia*. It enhances rhizobial survival and is widely used as an adhesive in inoculation of legume seeds. Vincent et al. (1962) found that gum arabic not only protected cells against desiccation on beads but resulted in better survival on seeds than maltose suggesting some protection against toxic seedcoat factors. However, the difference in adhesion to seed of these two additives was not considered. Variable quality, availability, cost and the need for high concentrations (15–40% w/v) has limited the use of gum arabic as an inoculant adhesive. Methyl cellulose, a non-ionic watersoluble cellulose ether, is a more widely used adhesive. It is readily available, its quality is relatively consistent as it is a semi-synthetic polymer and it is relatively low-cost due to the application of low concentrations (1.5% w/v) (Scott, 1989). However, there are variable reports on the protection of rhizobia by methyl cellulose when compared with gum arabic and it is generally considered to be less effective. Elegba and Rennie (1984) found no difference between survival, nodule mass and plant yield when rhizobia were applied to soybeans with methyl cellulose and gum arabic. However, the results are difficult to interpret due to the disparate nature and different concentrations of the two polymers. This is further confounded by comparisons between different *Rhizobium* and legume species.

On the other hands, water-soluble polyvinylpyrrolidone (PVP) was patented as a seed-coating agent by Lloyd (1979). PVP is a synthetic vinyl polymer produced by free radical vinyl polymerization of the monomer vinylpyrrolidone. Hale and Mathers (1977) demonstrated the water insoluble form of PVP defined as polyvinyl polypyrrolidone, PVPP, and water soluble form of PVP adsorbed toxic seed exudates from clover seeds. PVP can form insoluble complexes with phenols and the amounts bound were from 31 to 44% of the dry weight of polymer. PVP is a strong proton acceptor and therefore has a high capacity for binding phenolics. Moreover, phenolic binding was also observed in proteins such as casein and synthetic polyamides. Hale and Mathers (1977) demonstrated phenolic adsorption by several materials. In addition, antibiotic activity of toxic seed diffusates was also suppressed by activated charcoal and skim milk powder.

Evidently, the various additives applied during seed inoculation have provided some protection to rhizobia. However, polymers need to be selected carefully so that individual properties can be isolated and specific affects attributed to them. Desirable properties may then be optimized contributing to an overall positive effect on survival.

2.3.3 *Rhizobium*

One choice for development rhizobial inoculant production is improving at genetic level of rhizobia in order to have a better survival in stress conditions. Acid stress was selected for this study. Various mechanisms, including decreasing membrane permeability, amelioration of extracellular pH, proton extrusion/uptake, and prevention of metal ion toxicity may be involved in the tolerance to low pH

condition (Dilworth and Glenn, 1999). Gram-negative bacteria including rhizobia, are also known to enhanced level of tolerance to acidity, if cells are grown in moderately acid condition before exposure to higher acid condition (Goodson and Rowbury, 1989; Foster and Hall, 1990; Foster, 1991). The mechanism for this response has known as adaptive acid tolerance (ATR). Moreover, many genes have been identified involving in rhizobial survival in acid condition.

2.3.3.1 Genes involved in survival in acid condition

***actA*:** The mutant of *S. meliloti* carrying Tn*5* inserted in *actA* could not grow below pH 6.0 nor maintain a normal internal pH (pH_i) if the external pH (pH_e) is lower than pH 6.5 (O'Hara et al., 1989). The mutation in *actA* in *S. meliloti* also results in copper and zinc sensitivity; a complementing plasmid restoring the acid tolerance also corrects the heavy metal sensitivities. Homologues of the *actA* gene have been detected in all strains of *S. meliloti* tested, including acid-sensitive strains; while in acid-resistant strains expression of *actA* is constitutive with respect to pH (Tiwari et al., 1996*a*). The deduced ActA protein is basic; the N-terminal segment is hydrophobic and the C-terminal segment hydrophilic, suggestive of a membrane location. Its closest homology (69% similarity) is to the CutE protein, which involved in copper transport of *E. coli* (Rogers et al., 1991), itself a homologue of an apoprotein N-acyltransferase in *Salmonella typhimurium* (Gupta et al., 1993).

***actP*:** The link between acidity and heavy metal ions appears again with mutant strains *S. meliloti* RT3-27 and *R. leguminosarum* bv. *viciae* WR1-14. In a minimal medium containing 2.5 μmol/l added $CuSO_4$ these mutants fail to grow below pH 5.8 and 5.5, respectively, but if no copper is added both strains grow normally at

low pH, thus showing copper sensitivity dependent on pH. The deduced protein sequences from the mutated genes in the 2 organisms are very similar to one another and show extensive (44–49% identity) matches to P-type ATPases from *S. typhimurium*, *Proteus mirabilis*, *Synechococcus* and *Bacillus subtilis*, leading to the gene designation *actP*. Expression of an *actP* in WSM710 is markedly induced by copper at both pH 5.5 and 7.0, but for a constant concentration of added copper the induction is much greater at the lower pH, possibly reflecting greater availability of free Cu^{2+} mediated by acidity. No such induction of *actP* expression is observed with zinc, indicating a specificity of ActP for copper (Dilworth et al., 2001).

***exoR and exoH*:** In *R. leguminosarum* bv. *viciae* WSM710 a Tn*5* insertion in *exoR* (the regulatory gene for exopolysaccharide (EPS) synthesis) results in over-production of EPS and a mildly acid-sensitive phenotype (Reeve et al., 1997), clearly in conflict with the suggestion by Cunningham and Munns (1984) that there is a correlation between acid tolerance and EPS production. In *S. meliloti* WSM419 (acid resistant strain), its mutant with Tn*5* inserted into its *exoH* gene (WRR1) is both acid and zinc sensitive and, as would be expected (Leigh et al., 1987), fails to succinylate its EPS (Glenn et al., 1999).

***The actS–actR system*:** *S. meliloti* requires a contiguous sensor (*actS*)–regulator (*actR)* pair of genes for growth at acid pH (Tiwari et al., 1996b); mutations in either result in acid sensitivity. The deduced protein sequence for ActS shows evidence for 5 strongly hydrophobic domains in the N-terminal region that are likely to be in the membrane. As well as 3 hydrophilic domains are likely to be involved in signal recognition. The C-terminal end of ActS shows similarity to other sensory protein kinases; appropriate regions show the invariant histidine and asparagine

residues, together with the glycine-rich region of 2 parts separated by 20–50 amino acids typical of these sensors (Tiwari et al., 1996b). The regulator gene, *actR*, next to *actS*, codes for a protein containing the invariant aspartate and lysine residues (probably 30Asp, 73Asp and 123Lys) typical of this group of regulators; 73Asp in ActS corresponds to 57Asp in CheY, and may, by analogy, be the residue which is phosphorylated in ActS. Subsequent analysis of the ActR sequence has identified a helix-turn-helix motif NVSETARRLNMNRRTLQRILAK suggestive of a role in DNA binding and gene regulation. The question what genes are controlled via ActS–ActR has been addressed by creating random mutations with mTn*5*-GNm (a mini-transposon carrying a promoterless *gusA*; Reeve et al., 1999) and identifying those mutants showing β-glucuronidase activity that varies according to the presence or absence of a functional *actS* or *actR*. Partial or complete sequencing of the genes carrying the reporter insertions has allowed identification of a variety of functions ranging widely across the metabolic spectrum and indicating that the ActS–ActR system may involve in the photosynthetic bacteria, a global regulator as well as a requirement for acid tolerance (Fenner et al., 2000). Very few of the ActS–ActR modulated genes have any very obvious connection to metabolism likely to be significant for pH homeostasis.

***phrR*:** Located downstream from *actA* in *S. meliloti*, WSM419 is a gene whose protein product shows similarities to regulatory proteins coded by the y4WC and y4aM genes in *Rhizobium* NGR234 (Freiberg et al., 1997) and has a helix-turn- helix motif for DNA binding. Fusion of *lacZ* or *gusA* to this gene shows that its expression is induced 5-fold by low pH, but also by stressful concentrations of copper, zinc, ethanol or hydrogen peroxide expression of *phrR* is not modulated by the *actS*–

actR system. Inactivation of *phrR* by insertion of the interposon ΩKm into 2 different codons results in no change to nodulation or N_2 fixation with *M. murex*, or to laboratory acid tolerance (Reeve et al., 1998).

***lpiA*:** A range of low pH-inducible genes has been localized by mutagenising *S. meliloti* with mTn*5*-GNm and comparing β-glucuronidase activity at acid and neutral pH. This gene, unlike *phrR*, is apparently only induced as a response to acid pH, not by other stresses. Its regulation appears to be independent of both the *actS–actR* system and of *phrR* (Dilworth et al., 2001).

Thus knowledge of pH-stress survival mechanisms on the basis of gene regulation would be lead to the construction of hardier rhizobial strains that will be better survive in acidity soil and further improve crop yields.

2.3.3.2 Role of EPS on bacterial survival

Rhizobium might be improved to have a better survival in stress conditions by using other strategies. EPS has been reported as a universal barrier for bacterial protection, can be the other one choice for development at genetic level (Prakash, 2003). Since EPS has an important role for successful nodulation and also have many properties that promote rhizobial cell to survive in the soil environment as following.

Protection rhizobial cells from desiccation: Hypothesis is based on the idea that specialized EPS-rich microhabitat somehow favors microbial survival in soils. One of the most popular hypotheses is that EPS help soil microorganisms to survive desiccation. Survivals of mucoid strains were more than nonmucoid strains

(Table 2.7). So, it may imply that living in EPS-rich habitats confers the competitive advantage on microorganisms during desiccation (Roberson and Firestone, 1992).

There are many mechanisms by which EPS may create a favorable micro-environment for survival of desiccation. One possibility is that EPS maintain a hydrated microenvironment around microorganisms at low water potential, which is the condition effect to microbial activity. Activity has been found to be sharply reduced when microorganisms are exposed to low matrix potential. When the external water potential decrease in a drying soil, bacteria may retain water by increasing their internal solute concentration, or they may lose water to their surrounding (plasmolyze), which can result in cell death (Roberson and Firestone, 1992). However, EPS hold many times their weight in water at low water potentials. Higher water content in the immediate microenvironment could favor microbial survival in several ways. These include maintaining hydration of microbial membranes and extracellular enzymes and trapping water within the EPS matrix. EPS may also trap dissolved nutrients, increasing nutrient availability in the microhabitat and offsetting the decrease supply of nutrients by diffusion during drying. EPS also increase the outer surface area of the cell and the numbers of contact points with the soil matrix. Therefore, nutrients present in the vicinity of the microorganism may then also are able to diffuse to the cell (Chenu and Roberson, 1996). All this may help microorganisms to maintain physiological function and to partially compensate for the substrate limitation that occurs during desiccation.

Protection rhizobial cells from metallic cations: The interaction between microbial anionic polymers and heavy metals has important ecological and practical implication. It can be useful for removing toxic heavy metals from solutions.

Table 2.7 Survival of mucoid and nonmucoid strains

Strains	Mucoidy	Survival rate (%)
Escherichia coli		
JT4000	+	23.0
SG20247	+	21.0
SG20250	-	3.0
ZO4006	-	2.2
Erwinia stewartii		
DC283	+	5.1
DM14101	+	2.7
PJD1910	-	0.6

Modified from Roberson and Firestone (1992)

In *Rhizobium* or *Bradyrhizobium* also has anionic exopolysaccharide. Therefore, some work has been studied the binding of acidic exopolysaccharide produced by *Bradyrhizobium* strain and several metal cations (Corzo et al., 1994). *Bradyrhizobium* EPS was precipitated by Fe^{3+}, Tn^{4+}, Sn^{2+}, Mn^{2+}, Co^{2+} and Al^{3+}. The presence of Fe^{3+} increased the EPS precipitation by aluminum (Table 2.8).

In this regard, the most important cations would be Al^{3+} and Fe^{3+}, because they are very common in soils. Aluminium has been reported to be toxic for both the members of the family *Rhizobiaceae* and the plants. So, the complexation and precipitation of Al^{3+} by EPS could be a detoxifying mechanism that promotes survival of rhizobia in high metallic cations soil.

Table 2.8 Effect of $FeCl_3$ on the precipitation of EPS-aluminum complex

$FeCl_3$ (mM)	% recovered in the precipitate		
	EPS	Fe^{3+}	Al^{3+}
0	76.6	-	40.3
0.75	96.4	98.1	33.3
1.0	97.0	99.3	45.5
2.0	100	100	70.1
5.0	100	99.1	72.7

Modified from Corzo et al. (1994)

Protection rhizobial cells from stress induced by acid soil: This hypothesis has been studied by determine the relationship between EPS production and acid tolerance (Cunningham and Munns, 1984). The results showed that acid-tolerant isolates from legumes produced significantly more EPS than acid sensitive isolates (Table 2.9). Figure 2.1 also show the correlation coefficient of the linear regression between EPS produced and acid tolerance. This is significant at the 1% level. Although, the correlation coefficient was not high, but an acid tolerance tend to be increased when high amount of EPS was produced.

Therefore, EPS is the one of strategies, which uses for development new rhizobial inoculant to protect cells from environmental factors and to successful in N_2-fixation. As the composition of rhizobial EPS and their biosynthesis or regulations have been investigated. Then, genetic engineering approach can be used for improving EPS production, such as improving the regulation gene system in order to increase EPS production.

Table 2.9 Extracellular polysaccharide production of acid tolerant and sensitive strains of *Rhizobium*

Host plant	Acid tolerance	mg EPS (in 100 ml)
Cicer	T (pH 4.3)	104.8
	S (pH 6.4)	59.6
Phaseolus	T (pH 4.2)	74.0
	S (pH 5.3)	39.9
Leucaena	T (pH 4.2)	43.7
	S (pH 5.3)	15.6
Melilotus	T (pH 5.1)	43.0
	S (pH 6.4)	1.3

T = Acid tolerant; S = Acid sensitive; Modified from Cunningham and Munns (1984)

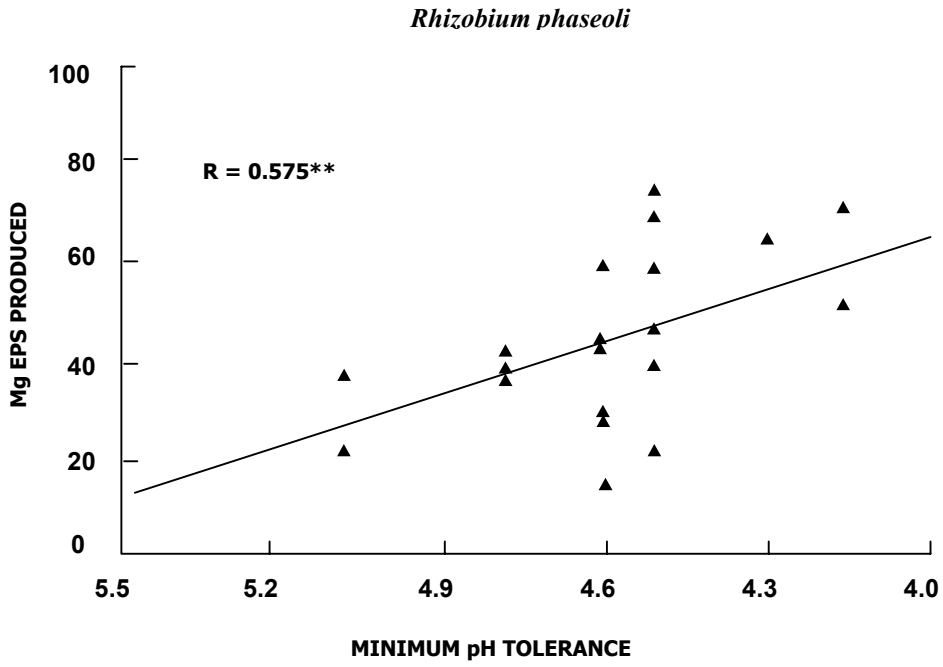

Figure 2.1 EPS production and acid tolerance of 20 strains of *R. phaseoli* (Cunningham and Munns, 1984)

2.3.3.3 Regulation of EPS biosynthesis

Various factors have been shown to regulate both the quantity and structural features of EPS. In *S. meliloti*, these factors include (*a*) osmolarity of the medium, which regulates the relative abundance of low- and high-molecular-weight forms of EPS I (Breedveld et al., 1990); (*b*) nitrogen starvation, which up-regulates EPS production and favors the production of low-molecular-weight EPS I forms (Dusha et al., 1999); and (*c*) phosphate limitation, which stimulates production of EPS, most clearly noticeable by a relatively higher production level of EPS II (Ruberg et al., 1999). Various regulatory genes that regulate synthesis of EPS I and EPS II have been identified in *S. meliloti* (Fig. 2.2).

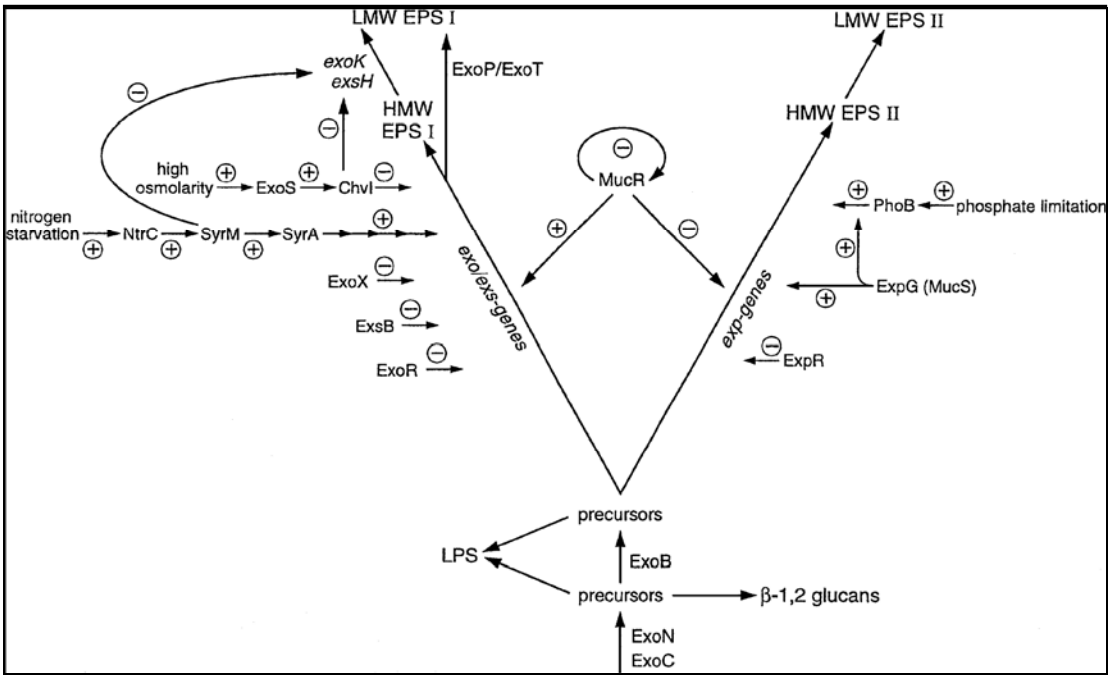

Figure 2.2 A model for the function of regulation of exopolysaccharide biosynthesis and depolymerization in *S. meliloti* (Spaink, 2000)

However, their interconnectedness is not yet understood. In *S. meliloti*, most notable is the role of the transcriptional regulator MucR protein, which is a key regulator of the relative levels of EPS I and II. The MucR protein, which is very similar to the transcriptional regulator Ros proteins of *Agrobacterium* spp. and *R. etli*, exerts its positive or negative regulation of the EPS biosynthesis genes probably directly by binding to a conserved DNA sequence called the Ros box (Spaink et al., 1998).

Other regulatory proteins are ExoR, ExsB, ExoR, ExoS/ChvI, ExpG, and ExpR. ExoR and ExoS are negative regulator, their mutant resulting in increase EPS production, but unable to nodulate or vary in N_2 fixation (Cheng and Walker, 1998; Gonz´alez et al., 1998; Dusha et al., 1999; Ruberg, 1999). Moreover, symbiotic regulator SyrM protein of *S. meliloti* is involved in regulating the relative ratios of low- and high-molecular-weight forms of EPS I. SyrM production is negatively regulated by SyrB (Barnett et al., 1998) and positively regulated by NodD3, which in turn is regulated by flavonoids and nitrogen limitation via NtrC (Dusha et al., 1999). This suggests many possibilities of co-regulation of Nod factor and EPS production. Recently, an alternative RNA polymerase sigma factor RpoH2 also found to involve in the regulation of EPS synthesis in *Rhizobium* sp. strain TAL1145 (Kaufusi et al., 2004). The *rpoH2* mutant in TAL1145 produces only 18% of EPS produced by TAL1145 wild-type. Even, genetic engineering approach can be used for improving EPS production. However, the effect of altering EPS production on legume nodulation and N_2 fixation must be concerned.

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CHAPTER III

CASSAVA AS A CHEAP SOURCE OF CARBON FOR RHIZOBIAL INOCULANT PRODUCTION USING AMYLASE-PRODUCING FUNGUS AND GLYCEROL-PRODUCING YEAST

3.1 Abstract

This research aims to develop methods to use low-cost carbon compounds for rhizobial inoculant production. Five raw starch materials; steamed cassava, sticky rice, corn, ground-dry corn and sorghum, were tested for sugar production by an amylase-producing fungus. Cassava produced the highest amount of reducing sugar after fermentation. Rhizobial cells from different genera were tested on medium supplemented with sugar obtained from cassava fermentation. All strains, except *B. japonicum* USDA110, could grow well in medium containing sugar derived from 100 g steamed cassava per liter. The derived sugar was further used for glycerol production using yeast. After yeast fermentation, the culture containing glycerol and heat-killed yeast cells, was used to formulate media for culturing bradyrhizobia. A formulation medium named FM4, with a glycerol concentration of 0.6 g/l and yeast cells supported growth of bradyrhizobia up to 3.61×10^9 c.f.u./ml in seven days. These results demonstrate that steamed cassava could be used to provide cheap and effective carbon sources for rhizobial inoculant production.

3.2 Introduction

For industrial production of rhizobial inoculants, it is important to identify inexpensive and easily available sources of nutrients for culture medium. In such media preparations, a single source of carbon cannot be used for all strains, because rhizobial strains of different genera often differ in carbon utilization. Generally, fast-growing rhizobia can utilize a variety of sugars such as glucose, sucrose, maltose, whereas bradyrhizobia appear to be nutritionally fastidious (Stowers, 1985). *Bradyrhizobium* can use arabinose, gluconate and some sugar alcohols, such as mannitol and glycerol as a preferred carbon source (Stowers, 1985; Lie et al., 1992). Mannitol is commonly used for cultivation both fast- and slow-growing rhizobia (Stowers, 1985).

For large scale production of rhizobial inoculant, it may be desirable to identify alternative sources of carbon that are less expensive than mannitol. Raw starch material can be changed to sugar by using biological processes (Chumkhunthod et al., 2001). Starch, for example, is composed of two polymers, α-D-glucose amylose and amylopectin, as linear and branch structures, respectively. Some bacteria and fungi produce α-amylase, β-amylase or amyloglucosidase (Zeikus and Johnson, 1991) that convert starch to maltose and glucose. It should be possible then to convert some inexpensive starch material, such as cassava tubers, sticky rice, corn or sorghum, into sugars by microbial fermentation for large-scale inoculant production of fast-growing rhizobia. Glycerol can be produced from glucose through yeast fermentation (Radler and Schutz, 1982; Parekh and Pandey, 1985; Vijaikishore and Karanth, 1987; Romano et al., 1997). Glycerol production using yeast fermentation can be enhanced

by adding sodium sulfite or alkaline reacting salts to the medium (James, 1928). Based on this information, we hypothesized that the unpurified sugars derived from microbial fermentation of low cost starch material could be used directly for producing glycerol, which could be further used as a carbon source for large-scale production of a bradyrhizobial inoculant. The objectives of this research were to select a suitable raw starch material and develop appropriate methods for sugar production using amylase-producing fungi, and glycerol production by yeast fermentation, and then use the unpurified sugar and glycerol to formulate a culture medium for cultivation of rhizobia and bradyrhizobia, respectively.

3.3 Materials and methods

3.3.1 Rhizobial strains

Fifty-two strains of bradyrhizobia (*Bradyrhizobium japonicum* USDA110 as a reference strain), and 9 strains of azorhizobia (*Azorhizobium caulinodans* IRBG23 as a reference strain) were obtained from the Department of Agriculture, Bangkok, Thailand. Six strains of rhizobia (*Rhizobium phaseoli* TAL1383 as a reference strain), 4 strains of sinorhizobia (*Sinorhizobium fredii* HH103 as a reference strain) and 4 strains of mesorhizobia (*Mesorhizobium ciceri* USDA2429 as a reference strain) were obtained from University of Minnesota, USA.

3.3.2 Media for testing carbon utilization

The basal medium for rhizobia contained 0.5 g K_2HPO_4, 0.2 g $MgSO_4.7H_2O$, 0.1 g NaCl and 0.5 g NH_4Cl per liter. Rhizobial strains were grown in basal medium

supplemented with various carbon sources, including succinate, malonate, tartrate, pyruvate, fumarate, gluconate, ribose, xylose, mannose, galactose, arabinose, cellobiose, raffinose, fructose, glucose, lactose, sucrose, propanediol, myo-inositol, sorbitol, glycerol, and mannitol. Each carbon source was prepared in 10% (w/v), and then filter sterilized aseptically before being added into the basal medium to a final concentration 1% (w/v).

3.3.3 Rhizobial cell preparation and cultivation on carbon utilization testing medium

Rhizobial starter cultures were grown in YEM until reaching 10^8-10^9 c.f.u./ml then washed twice with 0.9% (w/v) KCl (Stowers and Elkan, 1984). Five □□of cell suspension from several dilutions were dropped on agar media to determine growth on various carbon sources. The growth on different carbon sources was assessed after 7 days by comparing the colony development on basal medium without carbon sources amendment and YEM.

3.3.4 Raw starch materials and sugar production by amylase-producing fungi

Raw starch materials including fresh corn, ground-dry corn, diced cassava tuber, sorghum and sticky rice, were chosen to determine sugar production potential with amylase-producing fungus, *Chlamydomucor* SUT1 (amylase specificity 31.08 U/mg), which was previously described by Chumkhunthod et al. (2001). Pretreatment of raw starch material was done by steaming at 121°C for 5 min. SUT1 was grown on Potato Dextrose Agar (PDA) for 4 days, and 5 ml of sterile distilled water were added,

then the cell suspension was collected. Three ml of the mycelium suspension were added to 50 g of starch material, and fermented at room temperature for 7 days. The reducing sugars were determined every day by using the DNS method (James, 1995).

3.3.5 Formulation medium for rhizobia using sugar produced from selected raw starch material as the sole carbon source

Fermented cassava (100 g, 500 g and 1000 g) was mixed thoroughly with 1.0 liter of basal medium and the insoluble material was separated by centrifugation at 16,000 x g for 30 min. The supernatant containing the reducing sugar was adjusted to pH 6.8 and sterilized by autoclaving at 110°C for 40 min. The processed supernatants containing the three levels of reducing sugar were used as formulation media, FM1, FM2 and FM3.

3.3.6 Yeast culture

Yeast (*Saccharomyces cerevisiae*) isolate F3109 obtained from Dr. Chockchai Wanapu (Sripunya et al., 2005), was used for converting saccharified cassava into glycerol. The yeast culture was maintained as an agar slant in a medium modified from Lie et al. (1992). This medium contains 1.0 g urea, 1.0 g KH_2PO_4, 0.5 g $MgSO_4.7H_2O$, 2.5 g yeast extract and 100 g glucose per liter, and its pH was adjusted to 5.0 before autoclaving. To determine optimum conditions for glycerol production, the same medium was used with the following variations: (i) glucose was added as 10, 15, 20 and 40% (w/v) of medium; (ii) $CaCO_3$ was added in powder form as 3, 10, 15, and 20 g/l; and (iii) pH of fermentation was controlled either at pH 5.0 or 7.0 by an automatic pH controller with addition of 3 N NaOH and 3 N phosphoric acid, without supplement of $CaCO_3$. All media were sterilized by autoclaving at 110°C for 40 min.

3.3.7 Preparation of sugar solution from saccharified raw starch material for yeast fermentation

Selected raw starch material was saccharified by amylase fungus until the amount of reducing sugar reached maximum levels. One volume of distilled water was mixed well with two volumes of the saccharified raw starch material. The sample was centrifuged at 16,000 x g for 30 min. The supernatant was collected and diluted to obtain the optimum concentration of reducing sugar. Then, 1.0 g urea, 1.0 g KH_2PO_4, 0.5 g $MgSO_4.7H_2O$, and 2.5 g yeast extract were added per liter of this supernatant to obtain the yeast medium. The medium was buffered with $CaCO_3$ (3 g/l) and the pH was adjusted to 7.0. Media were sterilized by autoclaving at 110°C for 40 min.

3.3.8 Small scale yeast fermentation

A loop full of yeast collected from agar medium was inoculated into 125-ml Erlenmeyer flask containing 50 ml of normal medium as described above, and cultured on rotary shaker for 24 hours at 30°C, 200 rpm. This culture was used as inoculum. Ten milliliters of yeast inoculum was added to 90 ml of medium in 125-ml Erlenmeyer flask and incubated at 30°C for 7 days, without shaking. After fermentation, the medium was autoclaved at 110°C, 40 min to lyse cells, then centrifuged at 23,000 x g for 5 min. Supernatant was collected for glycerol determination.

3.3.9 Large scale yeast fermentation

One hundred milliliters of yeast inoculum was added to 900 ml of medium in a 1.5 liters fermentor. The temperature of fermentation was controlled at 30°C, and

culters agitated at 200 rpm. In an experiment to study the effect of pH on glycerol production (without $CaCO_3$), pH was controlled at 5.0 and 7.0 during fermentation by an automatic pH controller with addition of 3 N NaOH and 3 N Phosphoric acid. For glycerol production using saccharified cassava, $CaCO_3$ (3.0 g/l) was added to the medium and pH was adjusted to 7.0. The fermentation was operated as described above without automatic pH controlling.

3.3.10 Bradyrhizobial growth using glycerol derived from yeast fermentation of saccharified starch materials

The medium for bradyrhizobial cultivation composed of (in 1.0 l) 0.5 g NH_4Cl, 1.0 g KH_2 PO_4, 0.2 g NaCl, 0.2 g $MgSO_4.7H_2O$, 0.04 g $CaCl_2$ and 0.04 g $FeCl_3$, supplemented with 5 g glycerol and 1.0 g yeast extract (Lie et al., 1992). In this formulation experiment, sugar prepared from saccharified raw starch material was used for yeast fermentation, which was fermented until maximum glycerol concentrations were produced. The fermented medium was diluted to either 5% or 10% (v/v) with basal medium (without glycerol and yeast extract), and sterilized by autoclaving at 110°C, 40 min. These processed media were used as formulation media, FM4 and FM5. All growth experiments, media were adjusted to pH 6.8. The experiment was carried out in 125-ml Erlenmeyer flask containing 50 ml of medium. A starter culture (0.5 ml) was inoculated into the medium and cultured on rotary shaker for 7 days at 28°C and 200 rpm. The final cell concentration was determined by using a total plate count method. The formulated medium was used to grow bradyrhizobia in a 2,000 ml Erlenmeyer flask containing 1,000 ml of fermented medium.

3.3.11 Glycerol determination

Samples for glycerol determination were centrifuged at 23,000 x g for 5 min. Supernatant was collected and glycerol content was measured with a glycerol determination kit using an enzymatic analysis (Boehringer Mannheim).

3.3.12 Field experiment

Bradyrhizobium japonicum USDA110 and soybean variety SJ2 were selected for testing the efficiency of rhizobial inoculant produced from cassava modified medium compared with YEM medium, in a field experiment located at the Suranaree University of Technology farm. The experiment used a Randomized Complete Block design with four replications. Seeds were mixed with liquid inoculant before sowing. Sixty days after planting, the nodule number, plant dry weight and seed weight were determined. Analyses of Variance (ANOVA) and means comparison (Duncan Multiple Range Test -DMRT) were analyzed with the Statistical Analysis System (SAS, Computer Program, 1995).

3.4 Results and discussion

3.4.1 Carbon source for different rhizobia

To identify the sources of carbon that could be utilized by strains of different fast and slow growing rhizobia, twenty-two carbon sources including C-3, C-4, C-5, C-6, C-12, C-18 compounds and some sugar alcohols were tested. Most strains of *Rhizobium* and *Sinorhizobium* grew with most of these carbon compounds, whereas

Mesorhizobium, *Azorhizobium* and *Bradyrhizobium* strains had a more narrow range of acceptable carbon sources. Some strains failed to use some monosaccharides, disaccharides, and sugar alcohols (Table 3.1). It has been reported that the key enzyme, invertase was detected only in cell extracts of *S. meliloti*, *R. leguminosarum* and *R. trifolii* when cells were grown with sucrose, lactose or maltose (Martinez et al., 1974). Similarly, C-4 compounds, such as succinate are known to support fast growth of *S. meliloti* (Ucker and Signer, 1978). However, succinate is not widely used as a carbon source by bradyrhizobia (Stowers, 1985). While, *Mesorhizobium*, *Azorhizobium* and *Bradyrhizobium* could use C-3, C-5 and some sugar alcohol, but the ability to use these carbon sources varied from strain to strain. Mannitol is the most commonly used carbon source for all fast and slow growing rhizobia. Glucose and maltose, which were the breakdown products of starch, were utilized by most strains in genera *Rhizobium*, *Sinorhizobium*, *Mesorhizobium*, but these sugars were utilized by only a few strains of *Azorhizobium* and *Bradyrhizobium*. However, all *Azorhizobium* and *Bradyrhizobium* strains could catabolize glycerol, which was produced from glucose during yeast fermentation.

Therefore, these results confirmed that sugars (glucose, maltose) and glycerol produced from raw starch material can be used as a sole carbon source for rhizobial cultivation.

Table 3.1 Carbon utilization by rhizobial strain in different genus

Carbon sources	Percent of rhizobial strain able to use carbon source*				
	Rhizobium	*Sinorhizobium*	*Mesorhizobium*	*Azorhizobium*	*Bradyrhizobium*
Succinate	50.0	75.0	0.0	22.2	42.3
Malonate	33.3	25.0	25.0	22.2	0.0
Tartrate	33.3	75.0	25.0	11.1	21.2
Pyruvate	0.0	25.0	0.0	11.1	5.8
Fumarate	50.0	100.0	0.0	11.1	0.0
Gluconate	83.3	100.0	25.0	66.7	71.2
Ribose	100.0	100.0	25.0	55.6	15.4
Xylose	83.3	100.0	75.0	22.2	51.9
Mannose	100.0	100.0	0.0	11.1	15.4
Galactose	66.7	100.0	25.0	22.2	50.0
Arabinose	66.7	100.0	50.0	22.2	51.9
Cellobiose	83.3	100.0	0.0	33.3	13.5
Raffinose	100.0	100.0	50.0	22.2	7.7
Fructose	100.0	75.0	25.0	33.3	51.9
Glucose	100.0	100.0	50.0	22.2	13.5
Lactose	100.0	100.0	25.0	33.3	3.8
Sucrose	100.0	100.0	75.0	44.4	7.7
Propanediol	83.3	100.0	50.0	44.4	1.9
Mannitol	100.0	100.0	75.0	77.8	100.0
Sorbitol	100.0	100.0	25.0	33.3	19.2
Glycerol	83.3	100.0	25.0	77.8	42.3
Myo-inositol	100.0	100.0	0.0	11.1	1.9

*Number of strains were used in the experiment; *Rhizobium*, 6; *Sinorhizobium*, 4; *Mesorhizobium*, 4; *Azorhizobium*, 9; *Bradyrhizobium*, 52

3.4.2 Cassava is an inexpensive source for producing glucose and maltose for *Rhizobium* inoculant production

As an alternative to using glucose and maltose in the growth medium for *Rhizobium* inoculant production, we used starch, fermented with amylase-producing fungus, as a source of glucose and maltose. Five low-cost raw starch materials; steamed cassava, sticky rice, fresh corn, dry corn and sorghum, were tested for sugar production by amylase-producing fungus SUT1 (Fig. 3.1a). Among these raw starch materials, steamed cassava provided the highest amount of sugar (83.63 mg/g), which was four-fold higher than the amount of sugar obtained from fresh corn (<20 mg/g) after four days of fermentation.

Saccharification of cassava was more efficient than other raw starch materials. Pretreatment of raw starch material by steaming produced more oligosaccharide. Steaming the substrates may have enhanced growth rate of the fungus SUT1 which increased total amylase activity. It may be that treating substrates with steam also reduced microbial contaminants. Therefore, the concentration of reducing sugar was increased during the growth of SUT1. However, after four days the amount of reducing sugar decreased after 4 days of fermentation because some remaining heat tolerant microbial contaminant might have used sugar produced by the fungi (Fig. 3.1b) (Chumkhunthod et al., 2001). Therefore, steamed cassava was selected as the source of starch fermentation using amylase-producing fungus to obtain reducing sugars for rhizobial cultivation.

Different amounts of saccharified cassava were mixed well in basal medium in order to get different concentrations of sugar. The amount of reducing sugar 12.05, 70.45 and 220.10 mg/ml were detected in medium derived from 100, 500, and 1000 g

steamed cassava, respectively. These solutions were used for rhizobial growth experiment. The results indicated that the *Rhizobium, Sinorhizobium, Mesorhizobium* and *Azorhizobium* strains could grow in medium containing cassava sugar derived from 100 g steamed cassava per liter, and the growth rates for these strains were similar to those cultured in medium containing 0.5% (w/v) mannitol (Fig. 3.2). HPLC analysis of this medium before rhizobial inoculation showed that it contained 0.56% (w/v) glucose and 0.20% (w/v) maltose. When the amount of cassava sugar in the medium was increased to equivalent of 500 g of steamed cassava per liter, the rhizobial growths were not observed. The *Bradyrhizobium* strain did not utilize cassava sugar as a carbon source. Therefore, the next step for glycerol production from cassava sugar is required.

3.4.3 Cassava as a cheap source for glycerol production for *Bradyrhizobium* inoculant production

The effect of initial glucose concentrations on glycerol yield from yeast fermentation was determined. Results showed that the yield of glycerol was directly correlated with the glucose concentrations (R^2 = 0.88), the yield of glycerol increased when the concentration of glucose increased (Fig. 3.3a). Normally, ethanol and glycerol are formed in high amounts, when higher sugar concentration is used for fermentation (Radler and Schutz, 1982). A concentration of 10% (w/v) glucose in the medium, which produced a glycerol yield of 2.58 g/l or 25.8 mg/g of glucose, was selected for development of glycerol production in further studies, because such a concentration of glucose could be easily achieved through steamed cassava fermentation.

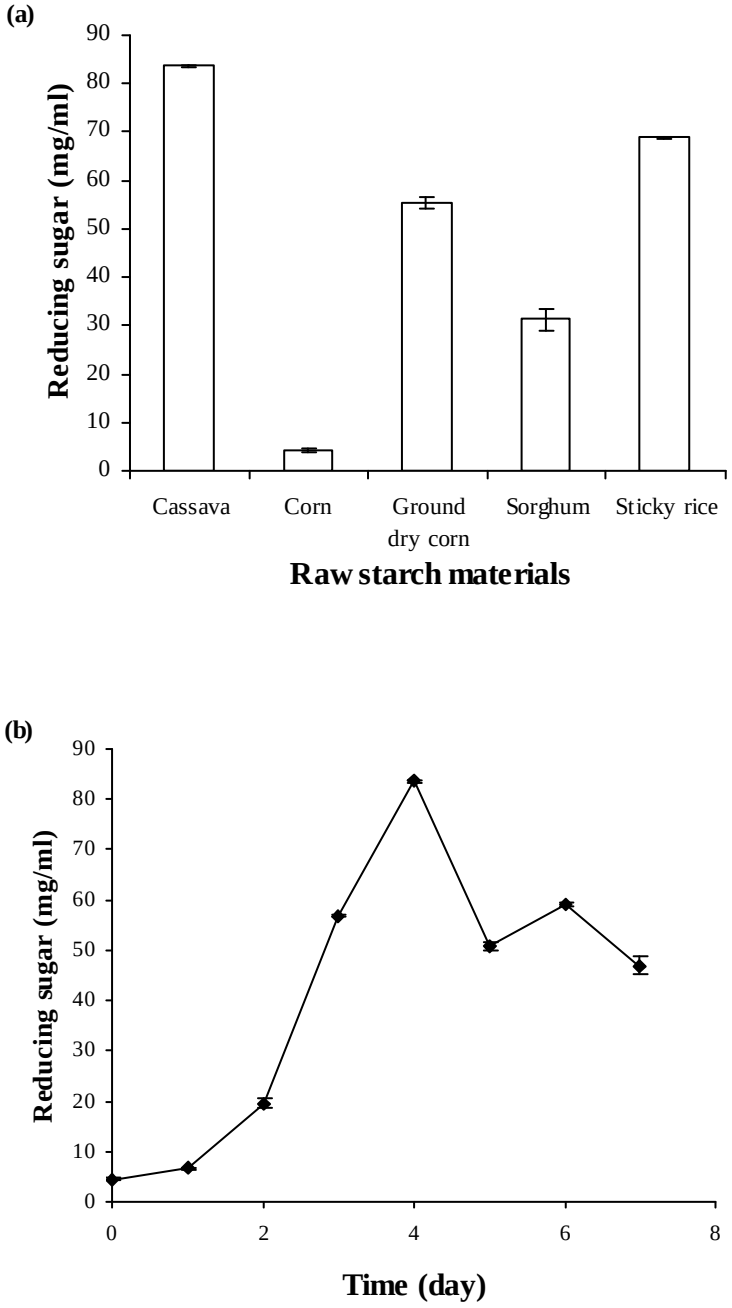

Figure 3.1 Reducing sugar produced from the fermentation of SUT1 with various raw starch materials (a), and time-course of reducing sugar production from the fermentation of SUT1 with cassava (b)

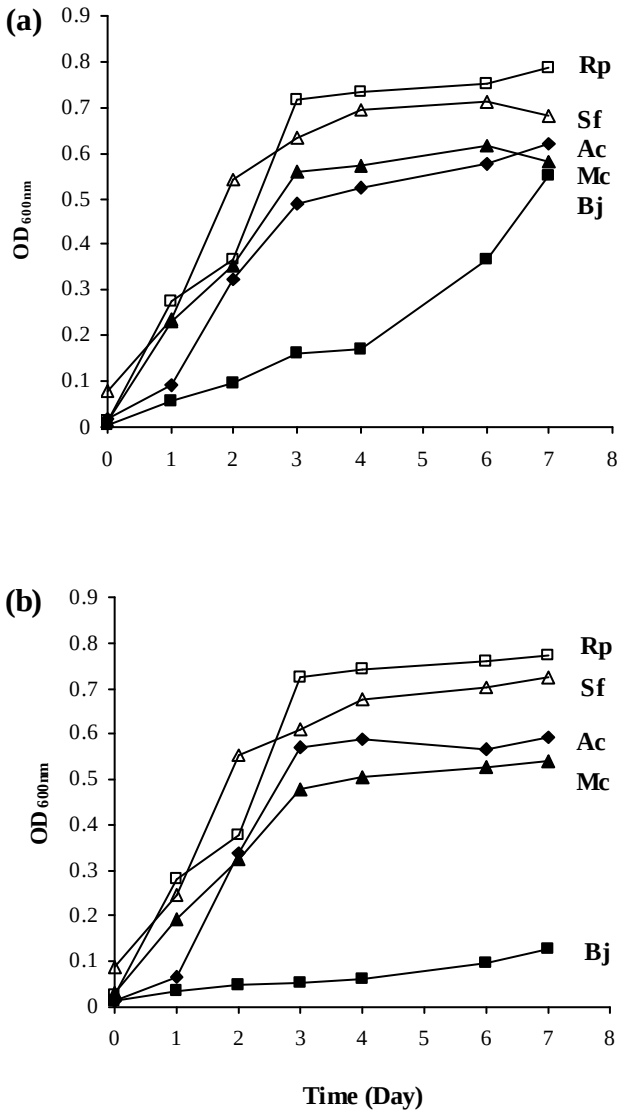

Figure 3.2 Growth of rhizobia in basal medium containing 0.5% (w/v) mannitol (a), and formulated medium made from 100 g of cassava per liter (b). Symbols used for different genera: *Azorhizobium* (closed diamond, Ac); *Bradyrhizobium* (closed square, Bj); *Mesorhizobium* (closed triangle, Mc); *Rhizobium* (open square, Rp); and *Sinorhizobium* (open triangle, Sf)

The effect of $CaCO_3$ on the yield of glycerol from glucose fermentation was also determined, since it was reported that the addition of $CaCO_3$ to the medium enhanced glycerol production (Lie et al., 1992). These results demonstrated that the addition of 3 g/l $CaCO_3$ to the medium during yeast fermentation produced a glycerol yield of 4.47 g/l, which resulted in 67% increase in glycerol production, compared to yeast fermentation without $CaCO_3$, which glycerol was produced 2.58 g/l (Fig. 3.3b). $CaCO_3$ was added to buffer pH of the system as organic acids were produced during fermentation. More dissolution of $CaCO_3$ resulted in increasing the pH of medium, which allowed high amount of glycerol production. However, the increasing of $CaCO_3$ concentration to 10 g/l or higher did not enhance glycerol production further.

Since the alkaline conditions can also enhance glycerol production from glucose fermentation (James, 1928; Vijaikishore and Karanth, 1987; Lie et al., 1992). Therefore, the optimum pH condition for production of glycerol from glucose fermentation using yeast was determined. *S. cerevisiae* F3109 produced low amount of glycerol at pH 8.0 because the cell density was also low at this pH. The selection of pH for fermentation also depends on the endurance of yeast strain (James, 1928). Therefore, pH 7.0 was selected for glycerol production, although the optimum pH for growth for *S. cerevisiae* isolate F3109 is 5.0. The pH of system kept constant during fermentation by automatic pH controller. The amount of glycerol produced was 43.07% higher in pH 7.0 than in pH 5.0 at 72 h after fermentation (Fig. 3.3c).

Based on the results of the above three experiments on the amounts of glucose and $CaCO_3$, and the pH of the medium, another experiment was conducted using sugar extracted from saccharified cassava. The cassava sugar solution was added to the growth medium to make the final concentration of sugar equivalent to 100 g/l,

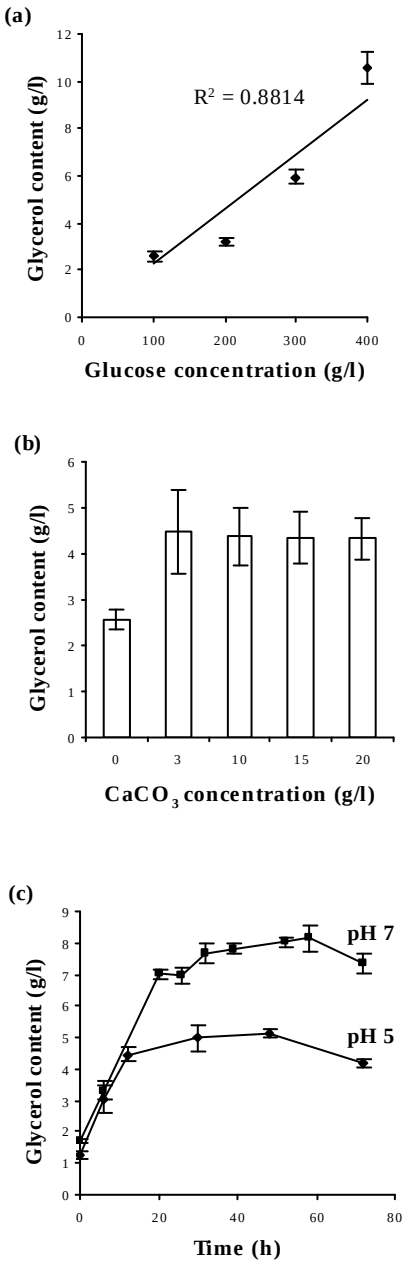

Figure 3.3 Glycerol production by yeast *Saccharomyces cerevisiae* isolate F3109 at different concentrations of glucose at pH 5.0 without addition of $CaCO_3$ (a), at different concentration of $CaCO_3$ at pH 5.0 (b), and without addition of $CaCO_3$ at pH 5.0 and pH 7.0 (c)

which is obtained from 1.2 kg of steamed cassava. $CaCO_3$ was added to this medium at a concentration of 3 g/l and the pH was adjusted to 7.0. Although the growth of *S. cerevisiae* reached a stationary phase at 12 h (Fig. 3.4a), the conversion of sugar to glycerol continued until about 21 h (Fig. 3.4b). The amount of reducing sugar was less than 5 g/l, and the glycerol content in medium remained at 12-13 g/l after 21 h. The highest glycerol yield was 13.36 g/l or 133.6 mg/g of reducing sugar at 59 h. The pH was slowly decreased from 7.0 to 5.8 during the first 12 h, and remained unchanged till the end of the cultivation (Fig. 3.4a). The yeast dry weight of 12.11 g/l was obtained after fermentation.

3.4.4 Formulation media for mass culture of bradyrhizobia

The fermented medium containing 1.2-1.3% (w/v) glycerol and yeast cells was autoclaved and used as culture medium for *Bradyrhizobium* inoculant production. Two formulation media, FM4 and FM5, were prepared by supplementing *Bradyrhizobium* basal medium with 5 or 10% (v/v) fermented stock solution, respectively. Then the final glycerol concentration and yeast amount became about 0.6 and 0.606 g/l (FM4); 1.2 and 1.211 g/l (FM5). *B. japonicum* USDA110 could grow well in both FM4 and FM5. After 7 days, the bradyrhizobial population number in FM4 and FM5 reached 3.61×10^9 and 4.67×10^9, respectively, compared to 1.82×10^9 c.f.u./ml in the basal medium supplemented with 5 g/l glycerol and 1 g/l of yeast extract, and 6.35×10^6 c.f.u./ml in the basal medium without glycerol and yeast extract (Fig. 3.5).

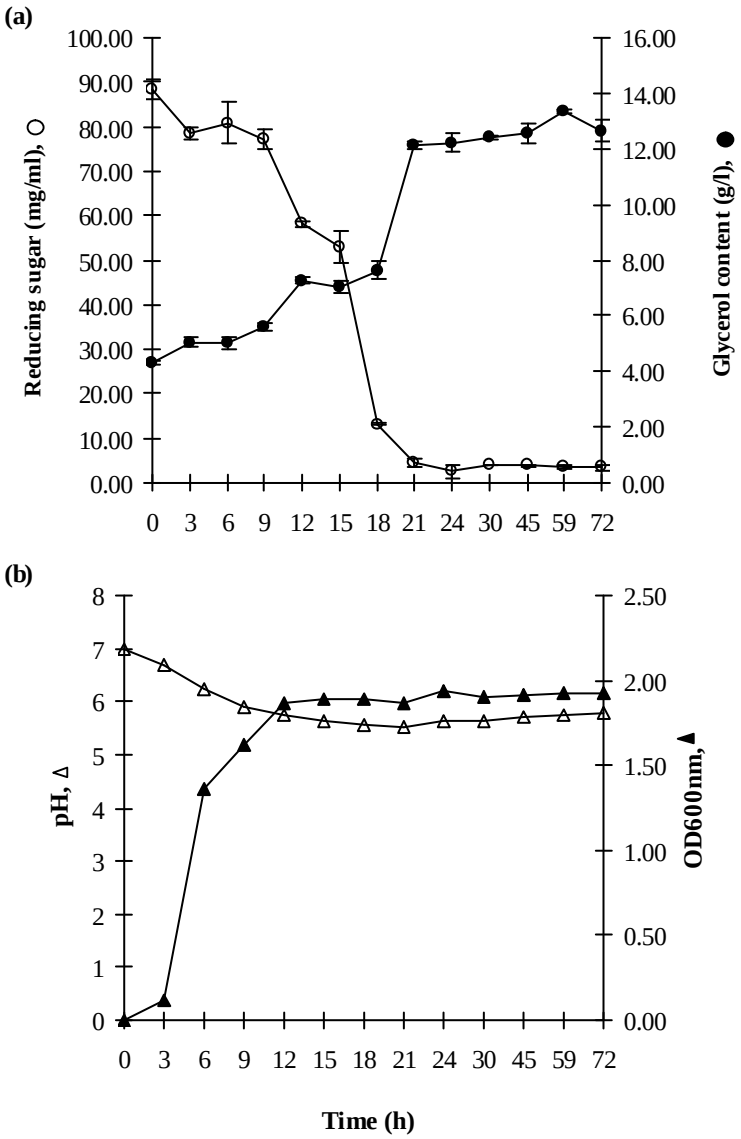

Figure 3.4 Time-course of sugar utilization and glycerol production (a), and growth of yeast *S. cerevisiae* isolate F3109 and subsequent pH changes in media containing sugar made from cassava (b). Symbols used for different parameters: reducing sugar (open circle); glycerol (closed circle); pH (open triangle); and OD_{600} (closed triangle)

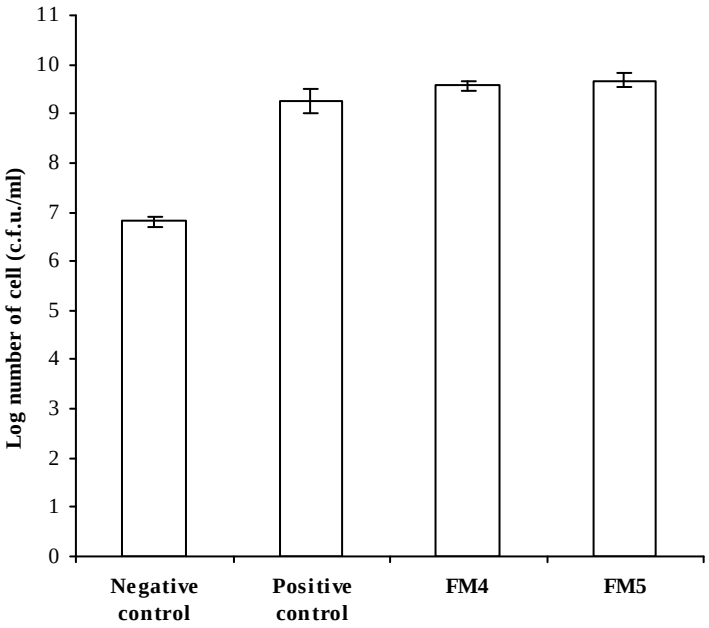

Figure 3.5 Number of viable bradyrhizobial cell in basal medium formulated from 5 and 10% (v/v) of yeast pre-fermented medium (FM4 and FM5), compared to basal medium containing 5 g/L glycerol as positive control, and basal medium without carbon source as negative control, after 7 days of cultivation

The FM4 was further tested for supporting bradyrhizobial growth using a 1.0 l culture volume. The number of cells and glycerol consumption by bradyrhizobia were determined. The glycerol concentration dropped from 0.65 g/l at the beginning of cultivation to zero after 5 days of cultivation. This indicates that glycerol was completely utilized by bradyrhizobia (Fig. 3.6). The number of bradyrhizobial cells reached 10^{10} c.f.u./ml after 6 days and decreased in one magnitude at one day after. Although the amount of glycerol in FM4 was only 0.6 g/l, FM4 also contained lysed yeast cells, which might provide additional source of nutrients, including vitamins and

amino acids. Thus, yeast cells from fermented medium could be used as a yeast extract supplement for bradyrhizobial cultivation.

Moreover, to evaluate the efficiency of rhizobial inoculant produced from cassava, the field experiments were also determined. The results showed that rhizobial inoculant derived from cassava could allow nodulation with soybean and produce soybean equivalent to YEM-based inoculant (Table 3.2).

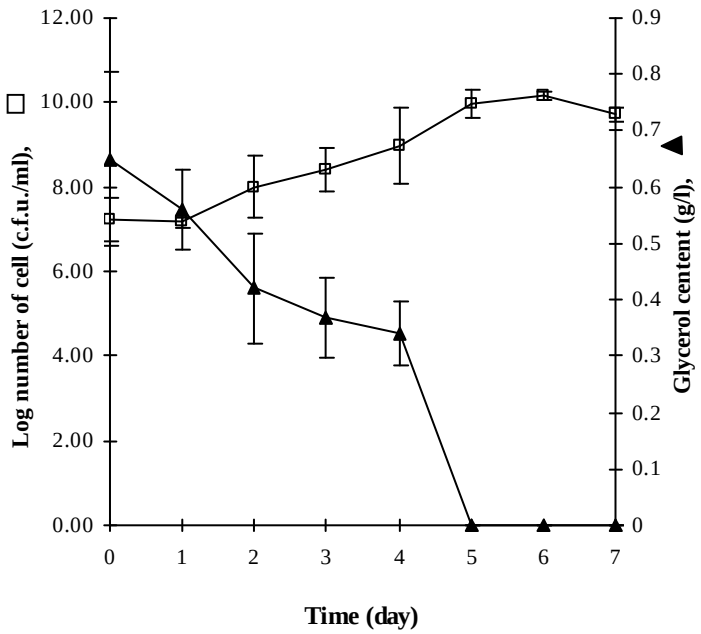

Figure 3.6 Time-course of bradyrhizobial growth and glycerol utilization in formulated medium FM4. Symbols used for different parameters: viable cell (open square); and glycerol content (closed triangle)

Table 3.2 Effectiveness test of *B. japonicum* USDA110 inoculant produced from cassava with soybean SJ2

Treatments	Nodule number (1 plant)	Plant dry weight (kg/rai)	Seed weight (kg/rai)
Control (uninoculate)	1 b	101.0 b	119.1 b
Mannitol (YEM)	35 a	119.6 a	185.6 a
Glycerol (derive from cassava)	44 a	164.9 a	206.1 a

Means with different letters are significantly different at $p<0.05$

3.5 Conclusion

The results revealed that cassava, a low cost starch material, could be used for rhizobial inoculant production. The first step of starch saccharification using amylase-fungi produced glucose and maltose, which were used by rhizobia in genera *Rhizobium*, *Sinorhizobium*, *Mesorhizobium* and *Azorhizobium* as carbon sources. Further fermentation of the saccharified starch using yeast produced glycerol, which was used as the source of carbon for bradyrhizobia cultivation. The disrupted yeast cells in the medium may have provided an additional source of nutrients for bradyrhizobia. Thus cassava could be an appropriate cheap source of carbon for rhizobial inoculant production using amylase-producing fungus and glycerol-producing yeast.

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CHAPTER IV

GROWTH AND SURVIVAL OF RHIZOBIA IN LIQUID INOCULANT FORMULATIONS WITH DIFFERENT ADDITIVES AND THEIR FIELD PERFORMANCE

4.1 Abstract

Carriers for legume inoculants must support rhizobia growth and survival and be easy to apply under field conditions. In this research we compared the performance of a standard solid carrier (peat based inoculant) to a liquid carrier containing 6 different additives i.e. cassava starch, polyethyleneglycol 3000 (PEG), polyvinylpyrrolidone (PVP), polyvinylalcohol (PVA), arabic gum and sodium alginate. Performance of the inoculant formulations was evaluated with several strains from the genera *A. caulinodans* IRBG23, *B. japonicum* USDA110, *M. ciceri* USDA2429, *R. phaseoli* TAL1383 and *S. fredii* HH103. Different genera of rhizobia survived differently in different carriers. In the peat carrier, the population of *R. phaseoli* TAL1383 and *S. fredii* HH103 at 6 month was only 10^7 cells/g peat, while *M. ciceri* USDA2429 and *B. japonicum* USDA110 had 10^8 cells/g peat. *A. caulinodans* IRBG23 maintained a population in peat of 10^9 cells/g peat for up to 6 months. In the liquid carrier containing cassava starch at 0.5%, *A. caulinodans* IRBG23 and *B. japonicum* USDA110 survived very well and maintained population number at 10^8 cells/ml up to 6 months of storage. *R. phaseoli* TAL138 grew moderately while *M.*

ciceri USDA2429 and *S. fredii* HH103 could not grow with this additive. In the liquid carrier containing arabic gum (0.1%), *A. caulinodans* IRBG23, *B. japonicum* USDA110 and *M. ciceri* USDA2429 survived very well and maintained up to 10^8 cells/ml at 6 months, while *R. phaseoli* TAL1383 and *S. fredii* HH103 failed to survive. Survival of rhizobia in liquid carrier containing PVP (2%) was satisfactory with all strains tested except *S. fredii* HH103 which did not survive in this carrier. In liquid carrier containing PEG (1%), *A. caulinodans* IRBG23 and *B. japonicum* USDA110 survived well in this carrier up to 6 months but *M. ciceri* USDA2429, *R. phaseoli* TAL1383 and *S. fredii* HH103 could not survive. In liquid carrier containing PVA (0.5%), *A. caulinodans* IRBG23 survived very well at 10^8 cells/ml up to 6 months. *M. ciceri* USDA2429 was satisfactory survival and better than *R. phaseoli* TAL1383 but *B. japonicum* USDA110 and *S. fredii* HH103 could not survive in this carrier. It could be concluded that to produce good quality of inoculant, the suitable carrier or additive substance for the target rhizobia should be selected. Moreover, the survival of inoculated rhizobia after applied to seed under 40°C was also conducted and found that peat could maintain the highest number of rhizobial cell on seed at 10^5 cells/seed after stored for 48 h. Whereas, liquid inoculant containing arabic gum, sodium alginate, PVP, cassava starch could maintain at 10^4-10^5 cells/seed, and PEG and PVA could maintain only at 10^3 cells/seed. However, all liquid carriers showed performance in the field, such as nodule number, plant dry weight and seed yield, as good as solid carrier.

4.2 Introduction

In a finely ground and neutralized state peat is the standard in the rhizobial inoculant industry for carrier material due to its high water holding capacity, a high surface area, and support rhizobial growth and survival. Peat is not available in many countries, especially in the tropics, and may be exhausted in the future (Smith, 1992). Many materials such as soil, clay, charcoal, bagasse, perlite and rock phosphate have been tested their properties for supporting the growth and survival of rhizobia (Stephens and Rask, 2000). Before they can be used to produce inoculant these carriers need additional processing, such as mining, drying, milling and neutralizing. These processing steps require significant investment in equipment and increase operation costs for small production operations (Somasegaran and Hoben, 1994). Moreover, solid carriers are difficult to process to consistent characteristics, and may not appropriate for using with planting equipment used on large scale of field operations (Singleton et al., 2002). Therefore, liquid inoculant is a form which has been developed to solve problems associated with processing solid carriers.

Characteristics of a good liquid inoculant include non toxicity, low cost, readily available uniform materials, non perishable materials, adaptable to normal cell culture conditions, nearly neutral pH or easily adjusted, amenable to nutrient supplements, rapid release of rhizobia in the soil, supports rhizobial growth and survival and is manageable in the mixing and packaging operation (Singleton et al. 2002; Smith, 1992). Various liquid medium have been used for rhizobial culturing, these medium normally consist of carbon, nitrogen and vitamins, which promote the growth of rhizobia. Some additives, however, can be added to liquid medium for

other advantages, such as cell adhesion to seed, stabilizer, detoxifying seed exudates, or supporting rhizobial survival. Many polymers have been used for inoculant production due to their ability to limit heat transfer, good rheological properties and high water activities (Mugnier and Jung, 1985). Polymers, such as methyl cellulose, gum arabic, polyvinyl pyrrolidone (PVP), alginate, polyacrylamide are normally used as adhesives to improve seed coating by solid based carriers, or use as rhizobial entrapped in micro-bead form (Deaker et al., 2004; Dommergues et al., 1979; Bashan et al., 2002). However, using polymers in these forms are inconvenient since it requires additional or specialized treatment during sowing (Bashan, 1998, Bashan et al., 2002). Therefore, polymers that are soluble in the liquid growth medium are preferable.

Polymers used in this study were selected based on their properties, such as being dispersible in water rapid release of rhizobia from the polymer matrix, non-toxic, and have a complex chemical nature that slows degradation by soil microorganisms (Deaker et al., 2004). The objectives of this research are to identify appropriate additive substances for liquid based carriers that can promote growth and survival of rhizobia, and have field performance equivalent to peat based inoculant.

4.3 Materials and methods

4.3.1 Microoraganisms

Rhizobia from different genera were used in this experiment. *B. japonicum* USDA110, *A. caulinodans* IRBG23 were obtained from Department of Agriculture,

Bangkok, Thailand. *R. phaseoli* TAL1383, *S. fredii* HH103, *M. ciceri* USDA2429 were received from University of Minnesota, USA.

4.3.2 Medium and liquid carrier formulation

Yeast Extract Mannitol medium (YEM) was used for normal culturing of all rhizobia (Somasegaran and Hoben, 1994). Six additive substances, PVP (K40; Sigma); PEG (3000; Sigma); PVA (Sigma); Arabic gum (Carlo); Sodium alginate (Carlo); and cassava starch were added into YEM medium to determine whether the additives had a toxic effect on rhizobia. All additives were obtained from Sigma, except cassava starch. The additives were blended with a basal medium by modifying the G5 formulation (Singleton et al., 2002). The modified G5 medium is composed of (g/l) 1.0 mannitol; 0.5 K_2HPO_4; 0.2 $MgSO_4.7H_2O$; 0.1 NaCl; 1.0 yeast extract; 1.0 glucose; 0.5 arabinose; 200 μM Fe-EDTA and 4 ml glycerol. The different additives in the G5 medium were then evaluated for their effects on cell survival, seed germination and field performance.

4.3.3 Toxicity testing

YEM medium were blended with different concentrations of additives: PVP at 1.0, 2.0, 3.0, 5.0% (w/v); PEG at 0.1, 0.5, 1.0, 5.0% (w/v); PVA at 0.1, 0.5, 1.0, 3.0% (w/v); Arabic gum at 0.1, 0.3, 0.5, 0.8% (w/v); Sodium alginate at 0.1, 0.2, 0.3, 0.5% (w/v); Cassava starch at 0.1, 0.5, 1.0, 3.0% (w/v). The experiments were conducted in 125 ml Erlenmeyer flasks containing 50 ml of amended medium. A 0.1% (v/v) late log phase culture of rhizobia from each genus was used as an inoculant for each test medium, and grown in incubator shaker at 28°C and 200 rpm for 6 and 13 days for

fast growing and slow growing strains respectively. Fast growing rhizobia were sampled at day 0, 2, 4, 6, 8; and slow growing rhizobia were sampled at day 0, 6, 9, 13 to determine total viable cells by plate count compared with growth in YEM without additives.

4.3.4 Testing shelf life of liquid inoculant and storage condition

Cells were grown in 2 l Erlenmeyer flasks containing 1.4 liters of modified G5 medium with the appropriate concentrations of additive. Cultures were oxygenated by continuously pumping air through a sub-micron filter and into the culture flask until maximum cell concentration was reached. Aliquots of 20 ml of concentrated cell culture were inoculated into sterile polypropylene bags and then the bag was sealed. These liquid inoculants were stored at room temperature (28-30°C) and the total viable cells were determined by plate count every month.

4.3.5 Inoculant survival on seed at high temperature

Liquid inoculant (2.0 ml) of *B. japonicum* USDA110 containing different concentrations of the additives or 0.5 g peat inoculant with 1.5 ml of water (Somasegaran and Hoben, 1994) was used to inoculate 100 g of soybean seed (SJ2 variety). Seed were coated in sterile 1.0 liter flasks and shaken for approximately 1.0 min until all seeds were uniformly wetted. Immediately following inoculation seeds in each treatment were divided in to 3 replicates, and incubated at 40°C. The number of rhizobia on seeds of each treatment was determined at 0, 24, and 48 h after inoculation. In each treatment, 10 seeds were removed from each replicate and transferred to test tube containing 10 ml of sterile 0.85% NaCl. The test tubes were

shaken vigorously for 5.0 min to wash the inoculum off the seed, and then 1.0 ml of resulting suspension was removed. Ten-folds dilution of the suspension were made and 100 µl of each dilution were spread in YEM-congo red agar medium containing 5.0 µg/µl of tetracycline as a selective marker for bradyrhizobia. The plates were incubated at 28°C for 7 days, when colonies on plates were counted.

4.3.6 Germination testing

Twenty ml of bradyrhizobial liquid inoculant containing different concentrations of selected additives were inoculated on 100 g seeds of soybean (SJ2) and gently mixed until uniformly wet. The inoculated seeds were separated into three samples and placed in moist vermiculite under artificial light for 3 days. The proportion of seeds germinated seeds was then determined.

4.3.7 Field experiments

B. japonicum USDA110 and soybean variety SJ2 were selected for field testing the performance of each carrier formulations. The field was located in Suranaree University of Technology farm. The peat based inoculant and a non-inocualted control were also included in the design. The field experiments were arranged in a Randomized Complete Block design with four replications.

Seeds were mixed with liquid inoculant before sowing. At 60 days from planting, the nodule number, plant dry weight and seed weight were determined. The data were analyzed statistically using the Statistical Analysis System (SAS, Computer Program, 1995). Analysis of Variance (ANOVA) and means comparison by Duncan's Multiple Range Test (DMRT) were applied to the data.

4.4 Results and discussion

4.4.1 Toxicity effect of additives on rhizobial growth

The effect of different liquid inoculant additives on the growth of rhizobia, sinorhizobia, mesorhizobia, azorhizobia and bradyrhizobia are shown in Figure 4.1-4.5. The results showed that some polymeric additives, such as PVP, cassava starch and arabic gum did not adversely affect rhizobial growth of any strains. Cells could grow well and some concentration of these polymers promoted better growth than in YEM. Some of polymeric substances, such as PVP have been blended with medium for normal culturing conditions of bradyrhizobia (Singleton et al., 2002), with no adverse effect on growth. The PEG, arabic gum and alginate have also been reported to be used with rhizobial inoculant as adhesive or solid microbead carrier (Bushby and Marshall, 1977; Temprano et al., 2002; Bashan et al., 2002), but there are no reports regarding to the effect of these materials on cell growth. There are no reports about the effects of PVA or cassava starch on rhizobial growth in culture media.

Since rhizobia do not use these polymers as an energy source (preliminary experiment), these polymers could have other properties that support cell growth. The PVP was used as commercial product, and using this product has been shown to increase the yield of fermented yeast. It is believed to detoxify the fermentation media by complexing phenolic compounds which may limit survival in the media. Moreover, PVP can reduce toxicity inherent in many active ingredients and can also bind to various dyes, drugs, and other chemicals (ISP, www, 2005). Therefore, it is possible that PVP might bind some toxic substances generated during cell growth. However, for other polymers there are no reports about their effects on cell growth.

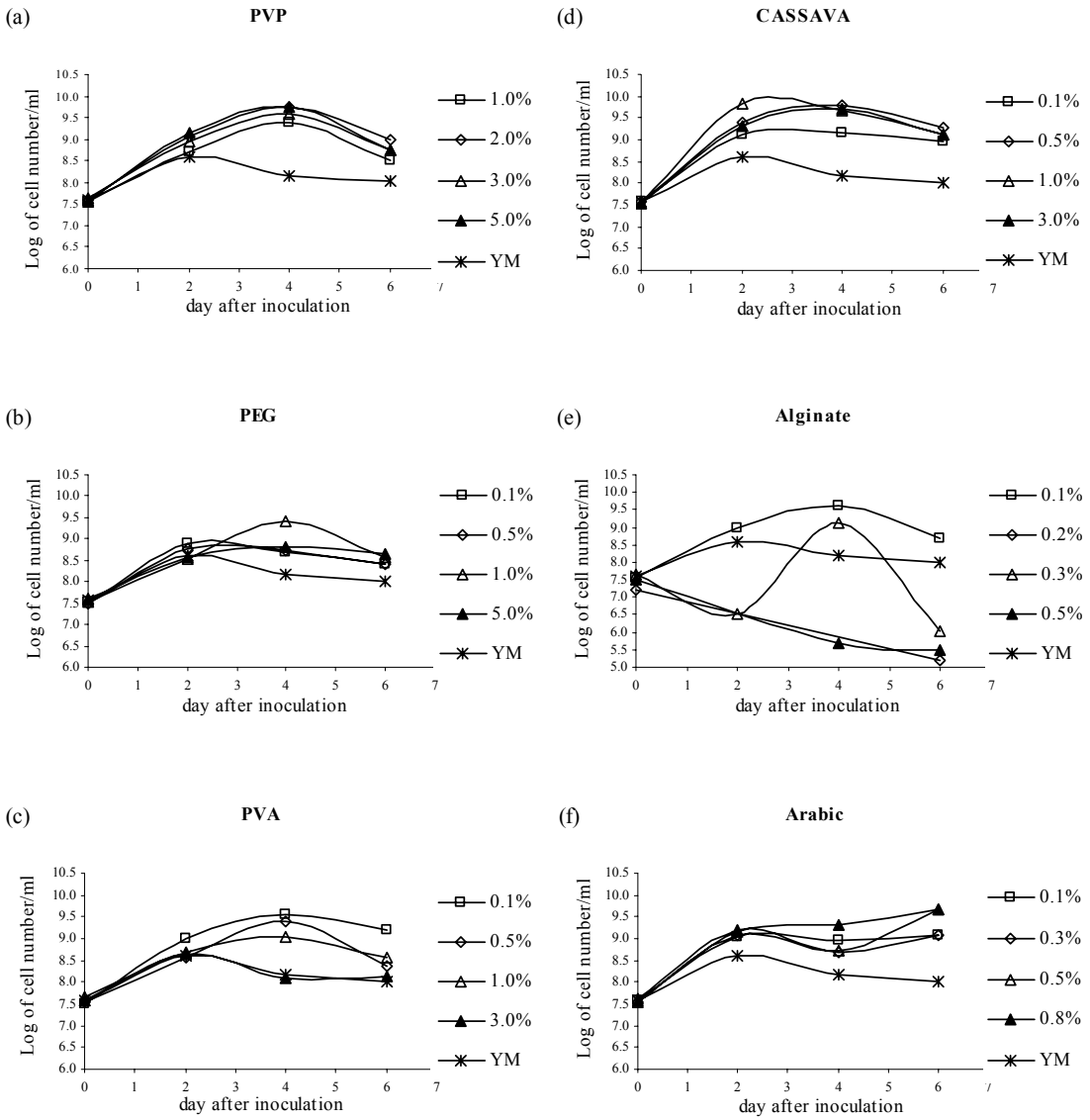

Figure 4.1 The growth of *R. phaseoli* TAL1383 in YEM medium blended with different concentrations (%w/v) of additives; (a) PVP; (b) PEG; (c) PVA; (d) cassava starch; (e) alginate; (f) arabic gum

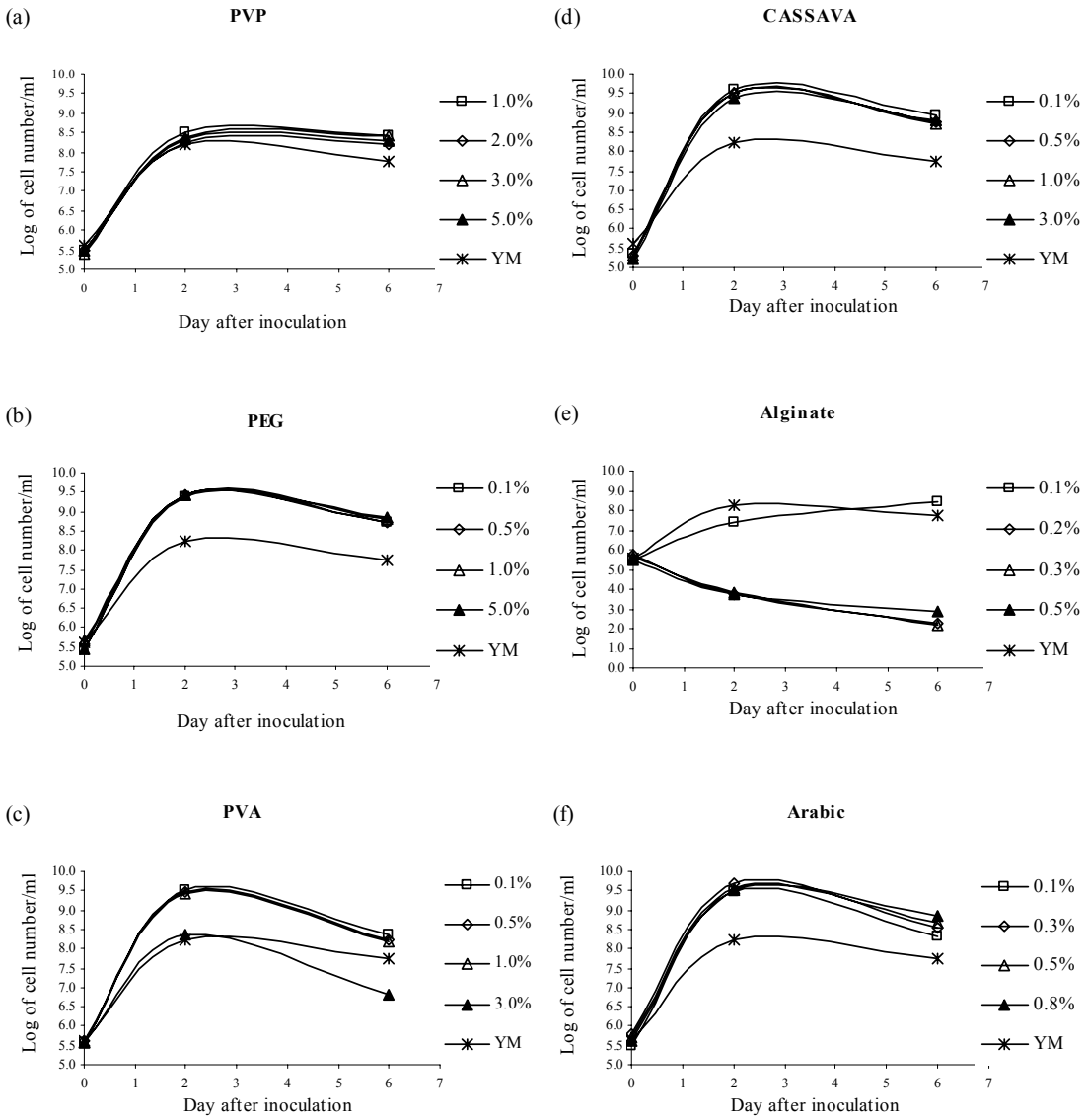

Figure 4.2 The growth of *S. fredii* HH103 in YEM medium blended with different concentrations (%w/v) of additives; (a) PVP; (b) PEG; (c) PVA; (d) cassava starch; (e) alginate; (f) arabic gum

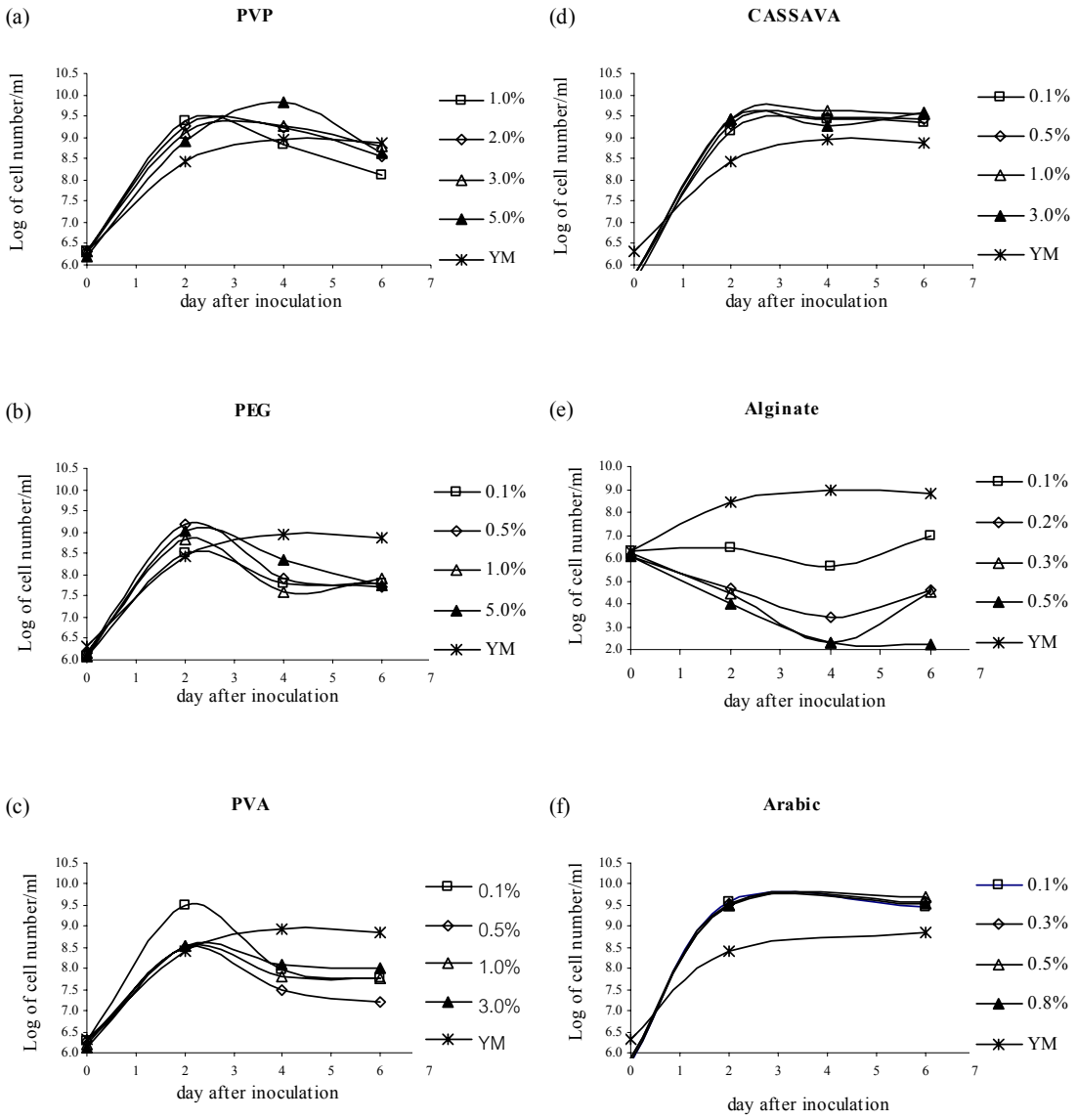

Figure 4.3 The growth of *M. ciceri* USDA2429 in YEM medium blended with different concentrations (%w/v) of additives; (a) PVP; (b) PEG; (c) PVA; (d) cassava starch; (e) alginate; (f) arabic gum

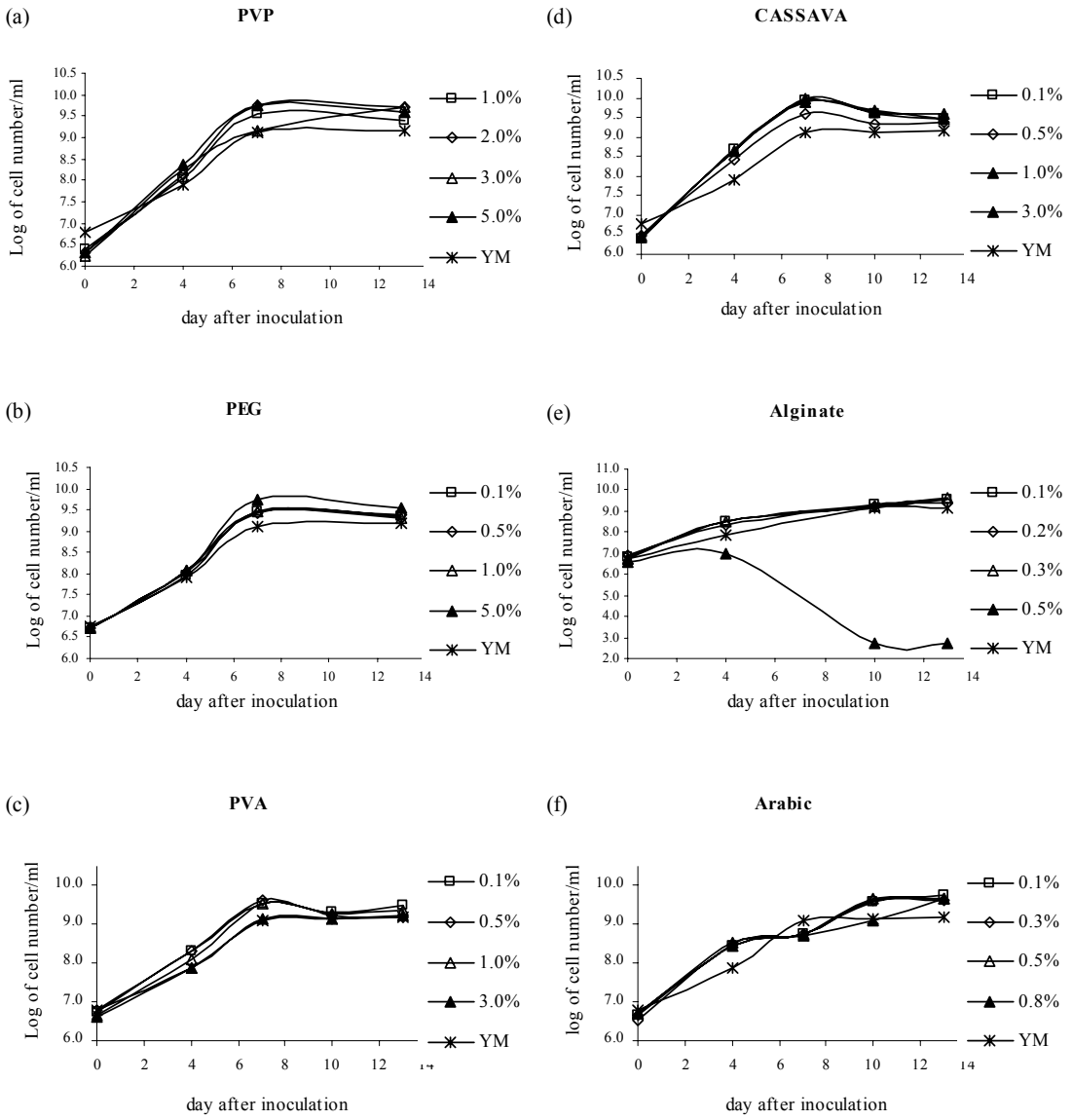

Figure 4.4 The growth of *A. caulinodans* IRBG23 in YEM medium blended with different concentrations (%w/v) of additives; (a) PVP; (b) PEG; (c) PVA; (d) cassava starch; (e) alginate; (f) arabic gum

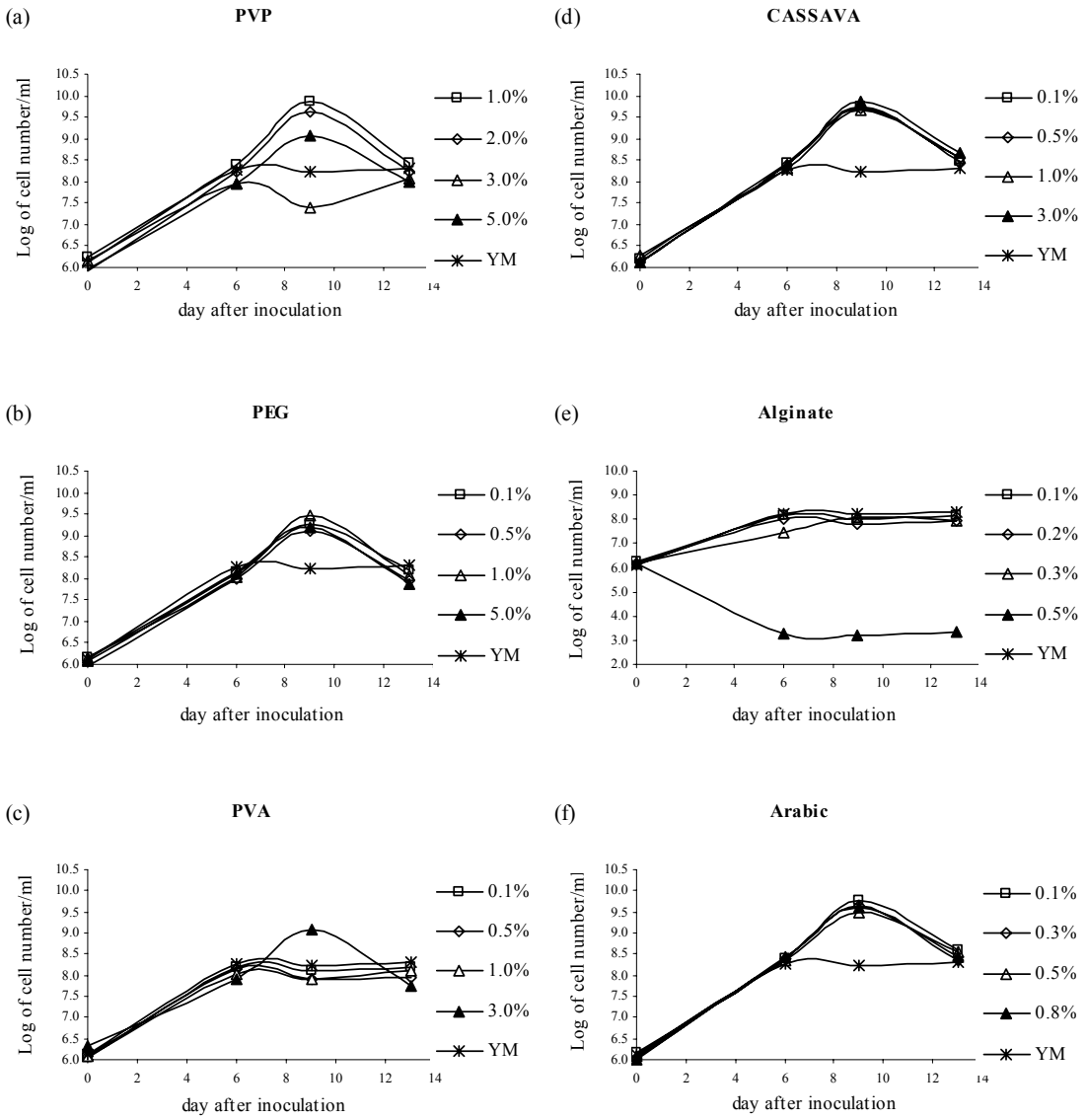

Figure 4.5 The growth of *B. japonicum* USDA110 in YEM medium blended with different concentrations (%w/v) of additives; (a) PVP; (b) PEG; (c) PVA; (d) cassava starch; (e) alginate; (f) arabic gum

The results showed PEG added to YEM promoted growth of all genera of rhizobia except *M. ciceri* USDA2429. Also PVA supported the growth of *R. phaseoli* TAL1383*, S. fredii* HH103 and *A. caulinodans* IRBG23, but some concentrations were not appropriate for *M. ciceri* USDA2429 and *B. japonicum* USDA110. In this case the final number of cells was less than in YEM medium. These results indicated that rhizobial strain and their sensitivity to foreign substances also influence on cell growth. Alginate seemed to reduce cell growth. It also showed an adverse effect on *M. ciceri* USDA2429. Increasing concentration of alginate in YEM medium to more than 0.1 % (w/v) reduced the number of *R. phaseoli* TAL1383 and *S. fredii* HH103 culture, but there was no effect on *B. japonicum* USDA110 and *A. caulinodans* IRBG23 until the concentration of alginate increased to 0.5 % (w/v). It might be possible that a high concentration of alginate reduced oxygen diffusion in medium.

Therefore, different concentration of additives, which were appropriate for each genus of rhizobia were selected based on their ability to promote cell growth (Table 4.1). These concentrations were used for testing the effect of polymeric additives on shelf-life of liquid inoculant and effectiveness of the additive on inoculant on performance in the field.

4.4.2 Survival of rhizobia in liquid inoculant formulation with polymeric additives

Another important aspect of rhizobial liquid inoculant is its shelf-life. The survival of rhizobia in different carriers is shown in table 4.2-4.8. Liquid inoculant containing PVP supported survival of *A. caulinodans* IRBG23 and *B. japonicum* USDA110 up to 6 months at cell concentration higher than 10^8 cells/ml. While cell numbers of *M. ciceri* USDA2429 and *R. phaseoli* TAL1383 were maintained at 10^6

Table 4.1 Selected polymeric additives used for formulating liquid inoculant

Carriers	Concentration of additives (%) used in each strain of rhizobia*				
	Azorhizobium	*Bradyrhizobium*	*Mesorhizobium*	*Rhizobium*	*Sinorhizobium*
PVP	2.0	1.0	2.0	2.0	1.0
	3.0	2.0	5.0	5.0	2.0
PEG	0.5	0.5	0.1	1.0	1.0
	5.0	1.0	0.5	5.0	5.0
PVA	0.1	0.1	0.1	0.1	0.5
	0.5	0.5	0.5	0.5	1.0
Arabic gum	0.1	0.1	0.1	0.5	0.3
	0.3	0.3	0.3	0.8	0.5
Cassava starch	0.5	0.5	0.5	0.5	0.1
	1.0	1.0	1.0	1.0	0.5
Alginate	0.1	0.1	0.1	0.1	0.1

*All additives were added directly to medium prior to inoculating the culture medium except sodium alginate which was added to medium after maximum cell concentration was achieved due to alginate obstruct the cell growth during culturing

cells/ml after 6 months of storage. In contrast, *S. fredii* HH103 did not survive in this carrier (Table 4.2).

Liquid inoculant containing PEG supported survival of *A. caulinodans* IRBG23 and *B. japonicum* USDA110 at cell concentrations higher than 10^8 cells/ml for 5 months, but PEG did not support survival of *M. ciceri* USDA2429, *R. phaseoli* TAL1383 and *S. fredii* HH103 (Table 4.3).

Inoculant containing PVA was found to support only the survival of *A. caulinodans* IRBG23, which had cell concentration more than 10^8 cells/ml during 6 months of storage (Table 4.4). However, PVA moderately supported the survival of *M. ciceri* USDA2429 and *R. phaseoli* TAL1383.

Table 4.5 demonstrates the survival of cells in liquid inoculant containing arabic gum. This polymer supported survival of *A. caulinodans* IRBG23, *B. japonicum* USDA110 and *M. ciceri* USDA2429 at up to 10^8 cells/ml for 6 months of storage, while *R. phaseoli* TAL1383 and *S. fredii* HH103 could not survive.

Inoculant containing cassava starch, maintained cell numbers of *A. caulinodans* IRBG23 and *B. japonicum* USDA110 at concentrations of more than 10^8 cells/ml for 6 months, respectively. Besides that, cassava starch could moderately support the survival of *R. phaseoli* TAL1383, but could not support the survival of *M. ciceri* USDA2429 and *S. fredii* HH103 (Table 4.6).

Interestingly, liquid inoculant containing Sodium alginate could support the survival of cell from every genus, especially *A. caulinodans* IRBG23, *B. japonicum* USDA110 and *M. ciceri* USDA2429, which remain the cell concentration at 10^7-10^8 cells/ml after 6 months. Also this carrier could support the survival of *R. phaseoli* TAL1383 and *S. fredii* HH103 at 10^5 cells/ml for 6 months of storage (Table 4.7).

Table 4.2 Survival of rhizobia in liquid inoculant containing polyvinylpyrrolidone (PVP)

Strains of rhizobia	Concentration of carrier (%)	Initial cell number (log no. of cells/ml)	Survival of cell from 1 to 6 months (log no. of cells/ml)					
			1	2	3	4	5	6
Azorhizobium	2.0	10.37	9.52	9.68	9.45	9.19	9.02	8.52
	3.0	9.68	9.52	9.63	9.74	9.10	9.05	8.40
Bradyrhizobium	1.0	10.07	9.76	9.62	8.97	8.92	8.91	8.76
	2.0	9.82	9.79	9.73	9.72	9.57	9.12	8.66
Mesorhizobium	2.0	9.12	8.64	8.14	7.94	7.41	7.23	7.08
	5.0	9.23	9.16	7.89	7.52	7.26	6.89	6.52
Rhizobium	2.0	9.23	8.75	7.95	7.81	7.44	6.51	6.38
	5.0	9.20	8.11	8.09	7.41	7.40	7.39	7.11
Sinorhizobium	1.0	9.67	6.29	4.15	3.02	0.00	0.00	0.00
	2.0	9.36	7.17	4.94	3.71	1.47	0.00	0.00

Table 4.3 Survival of rhizobia in liquid inoculant containing polyethyleneglycol 3000 (PEG)

Strains of rhizobia	Concentration of carrier (%)	Initial cell number (log no. of cells/ml)	Survival of cell from 1 to 6 months (log no. of cells/ml)					
			1	2	3	4	5	6
Azorhizobium	0.5	9.95	9.56	9.34	9.25	9.03	8.27	7.61
	5.0	10.06	8.79	7.64	7.24	7.02	6.59	6.37
Bradyrhizobium	0.5	9.74	9.56	9.13	8.36	8.28	8.09	7.86
	1.0	9.75	9.15	9.40	9.18	9.10	9.07	8.91
Mesorhizobium	0.1	< 6.00	ND	ND	ND	ND	ND	ND
	0.5	9.01	4.66	3.49	0.00	0.00	0.00	0.00
Rhizobium	1.0	9.80	7.14	3.00	0.00	0.00	0.00	0.00
	5.0	9.88	7.39	2.18	0.00	0.00	0.00	0.00
Sinorhizobium	1.0	9.13	6.04	5.83	3.75	0.00	0.00	0.00
	5.0	9.23	5.46	5.02	3.48	0.00	0.00	0.00

ND; not determine due to the initial cell number is less than 10^6 cells/ml

Table 4.4 Survival of rhizobia in liquid inoculant containing polyvinylalcohol (PVA)

Strains of rhizobia	Concentration of carrier (%)	Initial cell number (log no. of cells/ml)	Survival of cell from 1 to 6 months (log no. of cells/ml)					
			1	2	3	4	5	6
Azorhizobium	0.1	10.10	9.94	9.33	9.30	9.07	9.01	8.98
	0.5	9.86	9.38	9.26	9.10	8.78	8.62	8.61
Bradyrhizobium	0.1	10.07	5.74	4.80	4.51	1.56	0.00	0.00
	0.5	10.15	4.81	3.49	2.71	0.00	0.00	0.00
Mesorhizobium	0.1	9.21	8.76	8.52	8.06	6.44	6.36	6.21
	0.5	9.20	8.79	8.76	8.70	8.65	7.73	7.32
Rhizobium	0.1	9.06	7.88	7.51	7.42	5.11	4.62	4.60
	0.5	9.13	8.09	7.95	7.24	6.02	5.42	5.01
Sinorhizobium	0.5	9.35	8.94	4.28	0.00	0.00	0.00	0.00
	1.0	9.23	6.41	3.13	0.00	0.00	0.00	0.00

Table 4.5 Survival of rhizobia in liquid inoculant containing arabic gum

Strains of rhizobia	Concentration of carrier (%)	Initial cell number (log no. of cells/ml)	Survival of cell from 1 to 6 months (log no. of cells/ml)					
			1	2	3	4	5	6
Azorhizobium	0.1	9.93	9.81	9.74	9.69	9.45	9.06	8.48
	0.3	9.99	9.93	9.89	9.70	9.61	9.35	9.04
Bradyrhizobium	0.1	9.89	9.85	9.70	9.07	9.01	8.97	8.23
	0.3	9.98	9.79	9.78	9.77	9.67	9.60	8.53
Mesorhizobium	0.1	9.34	8.76	8.64	8.63	8.53	8.34	8.26
	0.3	9.07	8.38	8.36	8.16	7.94	7.52	7.52
Rhizobium	0.5	9.65	6.55	6.25	5.33	3.94	2.09	0.00
	0.8	9.03	6.84	4.97	4.07	3.98	1.96	0.00
Sinorhizobium	0.3	10.17	6.55	6.02	4.81	4.10	0.00	0.00
	0.5	9.18	5.97	5.90	3.57	3.50	0.00	0.00

Table 4.6 Survival of rhizobia in liquid inoculant containing cassava starch

Strains of rhizobia	Concentration of carrier (%)	Initial cell number (log no. of cells/ml)	Survival of cell from 1 to 6 months (log no. of cells/ml)					
			1	2	3	4	5	6
Azorhizobium	0.5	10.09	9.80	9.37	9.25	9.23	9.02	8.32
	1.0	9.90	9.29	9.25	9.09	8.98	7.57	7.31
Bradyrhizobium	0.5	9.94	9.81	9.00	8.41	8.07	8.05	7.97
	1.0	10.86	9.63	8.35	8.29	8.29	8.21	8.01
Mesorhizobium	0.5	9.06	5.01	3.43	3.09	0.00	0.00	0.00
	1.0	9.03	5.96	3.67	3.59	0.00	0.00	0.00
Rhizobium	0.5	10.01	9.53	6.64	6.25	5.36	5.12	4.31
	1.0	9.76	8.88	7.49	6.37	6.32	4.57	4.57
Sinorhizobium	0.1	9.16	7.37	6.89	6.40	5.38	5.07	1.14
	0.5	9.18	3.63	2.77	0.00	0.00	0.00	0.00

Table 4.7 Survival of rhizobia in liquid inoculant containing sodium alginate

Strains of rhizobia	Concentration of carrier (%)	Initial cell number (log no. of cells/ml)	Survival of cell from 1 to 6 months (log no. of cells/ml)					
			1	2	3	4	5	6
Azorhizobium	0.1	9.59	9.21	9.31	9.10	8.67	8.39	8.21
Bradyrhizobium	0.1	9.34	8.89	8.64	8.62	8.57	8.51	7.98
Mesorhizobium	0.1	9.02	8.87	8.30	8.10	7.53	7.52	7.48
Rhizobium	0.1	9.01	8.76	7.13	6.59	6.01	5.78	5.61
Sinorhizobium	0.1	9.30	7.15	6.85	6.73	5.87	5.32	5.01

Table 4.8 Survival of rhizobia in peat based carrier

Strains of rhizobia	Initial cell number (log no. of cells/g peat)	Survival of cell from 1 to 6 months (log no. of cells/g peat)					
		1	2	3	4	5	6
Azorhizobium	6.59	9.19	8.81	9.17	9.01	9.22	9.63
Bradyrhizobium	7.21	9.07	8.69	8.04	9.06	8.92	8.50
Mesorhizobium	7.21	8.98	8.96	8.75	8.45	8.00	8.38
Rhizobium	8.69	10.30	9.20	8.73	8.18	7.63	7.38
Sinorhizobium	6.44	9.30	8.45	9.21	9.22	9.22	7.90

Table 4.8 demonstrated that peat based carrier could support the survival of cell from every genus after 6 months at more than 10^8 cells/g and 10^7 cells/g for slow grower and fast grower rhizobia, respectively.

For commercial purposes a safe storage period of 6 months should be considered. Peat is wildly used in the final stage of the preparation of legume inoculants, and generally constitutes a suitable carrier for this purpose (Roughley and Vincent, 1967). It is common perception that rhizobia do not survive well in liquid inoculant especially when stored without refrigerator (Singleton et al., 2002). Maurice et al. (2001) reported the survival of rhizobia was mainly affected by O_2 availability and storage temperature. In the present experiment, storage temperature was 28-30°C, which could be an influence on the reduction of viable cells survive in all carriers. However, owing to the cost of building large cold storage rooms at the production plant, distributors and at the farmer in order to keep inoculant in refrigerator survival at normal room temperature is an important characteristic of inoculant formulations.

The results of storage demonstrated that peat could promote the growth of rhizobia. The number of cell increased after 1 month of storage, and still remains more than 10^7 cells/g peat after 6 months. While, the number of viable cells in most liquid inoculant formulations decreased more than in peat carrier during storage, especially *S. fredii* HH103 could not survive in any liquid inoculants very well. However, other strains such as *B. japonicum* USDA110 and *A. caulinodans* IRBG23 perform well in with many additives, while several additives perform well across a range of strain, such as PVP, arabic gum, and alginate.

It could be indicated that peat, which is rich in organic matter, could serve as energy source of rhizobia after liquid culture was injected into peat. In liquid

inoculant, rhizobial cells perhaps encountered starvation stress or nutrient depletion since the cells entry into stationary phase before packing in the container, and may cause the higher reduction of viable cell in liquid inoculant more than in peat. Moreover, polyhydroxybutyrate (PHB) may serve as an energy source for survival of rhizobia only in peat carrier, due to the lose of PHB in the cell after storage for 14 days peat carrier, while PHB still be observed in the cell stored in liquid medium for up to 14 days without shaking (Feng et al., 2002). However, the rhizobial cells in peat must also enter into a starvation condition when the energy source was finally exhausted. Feng et al. (2002) also reported that the nutrient limitation during storage induced cell wall thickening of rhizobial cell culture in peat, while no wall thickening occurred in rhizobial cell maintained in liquid inoculant at 30°C, but Maurice et al. (2001) found that the size and the shape of rhizobial cell were reduced when it maintained in liquid inoculant. Although the significance of these morphological changes was not established at the time, more studies with *Acinetobacter* and *Mycobacterium* have indicated that cell wall thickening may be related to enhanced survival of these cells under various types of stress (Cunningham and Spreadbury, 1998; Houang et al., 1998).

The rate of decline of viable cells differed between strains and formulation of liquid inoculant. There is a large interaction between strain and media components in determining of cell number in the inoculant (Singleton et al., 2002). The results showed that the survival of cell was dependent on both the type of additive and genus of rhizobia. In this experiment, alginate seemed to be the best polymeric additive substance, which could support the survival of cell from every genus more than 10^5 cells/ml after 6 month storage. The PVP also maintain the cell of rhizobia from every

genus more than 10^6 cells/ml for 6 months, except *S. fredii* HH103. While, liquid inoculant containing other additives could maintain only the survival of *B. japonicum* USDA110 and *A. caulinodans* IRBG23 at cell concentration more than 10^6 cells/ml after 6 months. Except PVA, although there was no toxicity effect during cell growth, but it seem to have adversely affect on *B. japonicum* USDA110 in long term survival. As slow-grower rhizobia normally can survive longer than 1 year even stored in water at 23-25°C (Crist et al., 1984). It means that some toxic substance might be occurred during storage of bradyrhizobia in inoculant containing PVA. However, the PVA and arabic gum could maintain the survival of *M. ciceri* USDA2429 at cell concentration more than 10^6 cells/ml after 6 months, while PVA and cassava starch could maintain the survival of *R. phaseoli* TAL1383 at cell concentration more than 10^6 cells/ml after 3 months. The reason why these polymers could maintain the survival of cell was not exactly known, but some polymeric additives such as PVP, PVA and starch have stabilization property. This protective has been known as colloidal stabilization. The improvement of survival is analogous to the protective colloid effect where bacteria represent one colloid and the suspension the others (Deaker et al., 2004). The polymer is absorbed in a thin molecular layer on the surface of the individual colloidal particles resulting in stabilize suspension and prevents coalescence of cells (ISP, on-line), which might block the O_2 and nutrients transferring from media to cells.

Slow growing rhizobia has been reported to survive longer than fast growing rhizobia both in liquid and peat carrier including in the soil (Crist et al., 1984; Mary et al., 1994; Bushby and Marshall, 1977). In liquid inoculant, rhizobia must be encountered with at least the nutrient starvation and O_2 depletion during storage. The stress conditions induced many changes of cell, such as change in cell morphologies;

reduce the cell division; reduce the level of protein, DNA, RNA synthesis to base level (Thorne and Williams, 1997). Therefore, the ability to establish a low rate of endogenous metabolism and the ability to utilize endogenous energy reserves are viewed as mechanisms by which bacteria maintain viability under stress conditions (Crist et al., 1984). This might be the reason why the production of liquid inoculant appears more appropriate for slow-glowing rhizobia than fast growers (Brockwell and Bottomley, 1995).

However, rhizobial inoculant should not be stored for a long time since excessive storage eventually induces physiological changes of rhizobial cells and changes colony appearance and nodulating capability including becoming more sensitive to desiccation. Therefore, the performance of rhizobial inoculant decreases with the amount of time the inoculant is stored (Catroux et al., 2001). It was confirmed in the field competition study in which an inoculant that was stored for one year gave a lower percentage of nodule occupancy than recently produced inoculant (Pinochet et al., 1993). Therefore, proper inoculant production management and distribution of inoculants should keep the time between manufacture and application to a minimum.

4.4.3 Survival of rhizobia on soybean seed at high temperature after inoculation

Inoculated seeds are normally sown into a furrow, which may expose the inoculant to temperatures at or above 40°C (Hafeez et al., 1991). Since temperature is known as one of the environmental factors affecting survival and nitrogen fixation of rhizobial inoculant (Hungria and Vargas, 2000), the role of polymeric additives on protection of rhizobial cells applied to seed was investigated and compared with

traditional peat-based carrier (Fig. 4.6). The results demonstrated that different additives had different abilities to protect rhizobial cells on seed at high temperature. The number of viable cells on inoculated seed decreased with time after inoculation. Peat-based carrier maintained the highest number of viable rhizobial cells on seed at about 10^5 cells/seed or 89.60% of cell survival after 48 h. Liquid inoculant containing arabic gum, sodium alginate, PVP, and cassava starch could maintain the number of rhizobial cell on seed about 10^4-10^5 cells/seed or remain 74%, 71%, 66%, and 56% of cell survival after 48 h, respectively. Liquid inoculants containing PEG or PVA maintained the number of cells surviving on seed at 10^3 cells/seed or less than 50% of applied cells survived 48 h after inoculation.

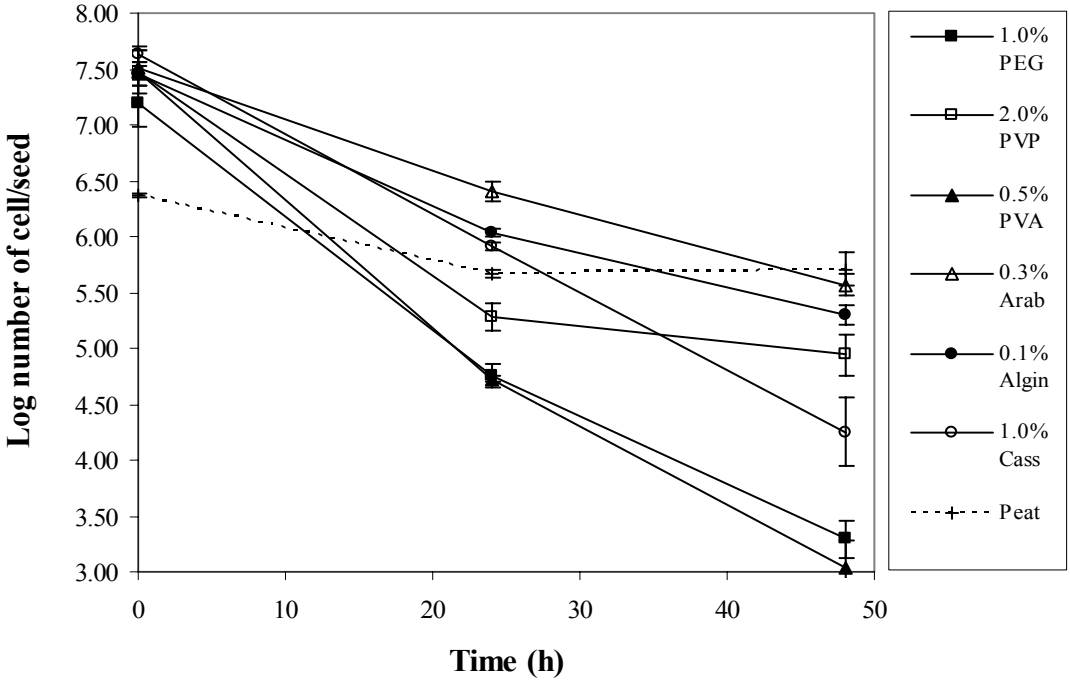

Figure 4.6 Survival of *B. japonicum* USDA110 on SJ2 soybean seed when peat and different liquid inoculants were inoculated to seed and storage at 40°C for 48 h

4.4.4 Effect of polymeric additives in liquid inoculant formulations on soybean seed germination and field performance

Liquid inoculant can be applied onto seed or in the furrow. When applied directly to seed, polymers in the inoculant solution tend to coalesce into ridges on the seed coat as the inoculant dries. This action may provide a localized thicker layer of polymer on the seed coat and affect seed germination. Polymeric additives were inoculated on the soybean seed, and the results of seed germination were evaluated. Results are shown in Table 4.9.

Table 4.9 The germination of soybean seeds inoculated with peat and liquid inoculant containing different polymeric additives

Carriers	Germination (%)
Control (without inoculation)	92.92 ± 4.56 a
Peat	89.69 ± 5.01 a
PVP (2% w/v)	88.33 ± 2.43 a
PEG (1% w/v)	80.91 ± 5.05 a
PVA (0.5% w/v)	87.21 ± 3.99 a
Arabic gum (0.3% w/v)	87.98 ± 8.50 a
Cassava starch (1% w/v)	84.02 ± 12.74 a
Alginate (0.1% w/v)	88.41 ± 10.56 a

Means ± S.D. with different letters are significantly different at $p<0.05$

Neither peat based inoculant nor liquid inoculant containing polymeric additives had an effect on the germination of soybean seeds. These liquid inoculants were then used for field experiment and evaluated their performance on number of nodules produced, plant dry weight and seed weight compared a control and the peat based inoculant (Table 4.10). The results revealed that liquid inoculant formulation with all polymeric additives could allow the nodulation with soybean and provide the yield of soybean as high as using peat based inoculant.

Table 4.10 Effectiveness test of *B. japonicum* USDA110 inoculant produced from different carriers with soybean SJ2

Carriers	Nodule number (1 plant)	Plant dry weight. (kg/rai)	Seed weight (kg/rai)
Control	2 c	158.92b	228.76 b
Peat	23 ab	223.68 a	301.28 a
PVP (1% w/v)	29 ab	227.84 a	309.76 a
PEG (1% w/v)	36 ab	248.96 a	322.28 a
PVA (0.5% w/v)	22 ab	221.56 a	288.48 a
Arabic gum (0.5% w/v)	30 ab	245.36 a	310.72 a
Cassava starch (1% w/v)	40 a	285.60 a	339.64 a
Alginate (0.1% w/v)	33 ab	251.60 a	328.28 a

Means with different letters are significantly different at $p<0.05$

These results confirmed the ability of polymers to protect rhizobial cells on seed after inoculation. Since it has been reported that desiccation, temperature and seed coat toxicity influenced the survival of rhizobia on seed (Taylor and Lloyd, 1968; Vincent et al., 1962), various polymers such as PVP, PEG and arabic gum have been used to enhance cell adherence to seed, slow drying of the inoculant after application and to protect seeds from desiccation.

Bushby and Marshall (1977) found the addition of PVP provided better protection of both fast- and slow-growing strains of rhizobia whereas PEG only protected fast-growing strains. PVP also has a high water binding capacity, which could maintain water around the cell (Singleton et al., 2002; Deaker et al., 2004). PVP and arabic gum has been reported to protect cells against toxic seed coat factors since PVP has high capacity for binding phenolic compounds released from the seed coat (Vincent et al., 1962; Hale and Mathers, 1977).

Besides that, as concentrations of salt increase in the cell environment under desiccation stress, stabilizing polymers, such as PVA may be useful in reducing the extent of protein precipitation or coagulation of cells. Maintenance of macromolecular structure may improve biological integrity thus leading to improve survival (Deaker et al., 2004). Moreover, biopolymers such as cassava starch, alginate and arabic gum have ability to limit heat transfer and high water activities (Mugnier and Jung, 1985). These might be the mechanisms that could improve the survival of rhizobial cell on the seed and gives rise to nodulation and nitrogen fixation as good as using peat based inoculant.

4.5 Conclusion

The results of this research indicate that many synthetic polymers such as, PVP, PEG, PVA, including biopolymers such as cassava starch, arabic gum and alginate can be used as additives to liquid inoculants. Since, certain polymers are able to mix in broth medium and cultures can be grown in their presence these aterials can be easily used in the production of liquid inoculant products. However, the shelf-life of liquid inoculant was shorter when stored at room temperature than peat based inoculants. This was especially true for fast-growing strains. Therefore, proper timing of inoculant production and distribution is more important for liquid inoculants than peat based inoculants. Results indicate that polymeric additives must be selected to match particular strains to optimize performance in liquid inoculants. Liquid soybean inoculants, manufactured with a range of polymers can improve the survival of rhizobial cells on the seed and promote nodulation and yield of legume that is equivalent to good peat based inoculant.

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CHAPTER V

EFFECTS OF TOXINS IN SEED EXUDATES FROM THAI SOYBEAN ON GROWTH OF RHIZOBIA

5.1 Abstract

Five varieties of Thai soybean, SJ4, SJ5, ST2, CM2 and CM60, were used to investigate whether the presence of the toxin in seed exudates affected the survival of four strains of bradyrhizobia. The toxicity effects of seed exudates on bradyrhizobia growth varied by soybean variety and the sensitivity of bradyrhizobial strain. Exudates from soybean variety CM2 caused the greatest inhibition of rhizobia growth and this exudates was then used to screen strains for sensitivity to seed exudate. Most bradyrhizobia and all rhizobia from other genera could grow in broth medium containing extracted seed exudates derived from 10 seeds, but growth was inhibited when the concentration of seed exudates increased by three and five folds. This revealed that the toxicity occurred when the amount of exudates was at high concentration, and the toxicity was non-strain specific. EPS was removed from *B. japonicum* TAL99 to determine the role of EPS on protecting cells from toxins in seed exudates. Growth of cells with EPS present showed no effect of seed exudates. When cells were washed to remove EPS cell growth was inhibited. The transcriptional regulator syrB (AraC family) was shown to have possible involvement in conferring tolerance to the toxicity of exudates.

5.2 Introduction

Exudates are compounds of low molecular weight that diffuse non-metabolically from intact plant cells into the surrounding environment. Diffusion of soluble exudates from seed is driven by a concentration gradient between the cell and external environment (Maier et al., 2000). Some people call the exudates diffusates (Bowen, 1961; Dadarwal and Sen, 1973). Exudates are composed of a wide range of compounds, such as sugars, amino acids, peptides, enzymes, vitamins, organic acids, nucleotides, carbohydrate, lipid, fungal stimulators, inhibitor, attracting factors and miscellaneous compounds including volatile compounds. Some of these compounds can physiologically stimulate or inhibit organisms (Maier et al., 2000; Rovira, 1969; Vancura and Stotzky, 1976). Therefore, the exudates from plants have an important role in the soil ecosystem. Fro example, carbohydrates and amino acids are important substrates for organisms, organic acids and lipids reduce pH of the rhizosphere and chelation of metal (Curl and Truelove, 1985).

Soybean seeds were used as model for this study. Ali and Loynachan (1990) investigated the toxic compounds in soybean seed varieties, Corsoy 79 and BSR 101. Their results indicated that soybean seed contains substances, which are toxic to bradyrhizobia. The toxins are soluble in water, thermostable and had a molecular size less than 2,000 Daltons. Genistein and daidzein were detected in high amount from soybean seed-exudates by using HPLC (Kape et al., 1992). Both genistein and daidzein are isoflavonoids, which were classified as phytoalexins (D'Arcy Lameta, 1985). Therefore, previous studies indicated the toxic compounds from soybean seed-exudates that are toxic to rhizobia belong to a group of phenolic compounds. These

phenolic compounds diffuse from the seed anytime the seed coat becomes wet (Bowen, 1961). The presence of toxic substances in the seed may limit the success of inoculation as it reduces the initial number of rhizobia on seed.

Many soybean varieties have been recommended to use for a field production in Thailand by the Department of Agriculture, such as Sor Jor 4 (SJ4); Sor Jor 5 (SJ5); Nakhon Sawan 1; SukhoThai 1 (ST1); SukhoThai 2 (ST2); Chieng Mai 2 (CM2); Chieng Mai 3 (CM3); Chieng Mai 4 (CM4); and Chieng Mai 60 (CM60) (DOA, www, 2005). To produce the rhizobial liquid inoculant for these soybeans, the effect of soybean seed exudates on rhizobia is one factor that needs to be considered. This research aims to determine whether the seed of Thai soybean varieties contain toxic substances that affect rhizobia growth and survival, and how rhizobial cells protect themselves from the toxin(s).

5.3 Materials and methods

5.3.1 Rhizobial strains

Bradyrhizobium japonicum strains USDA 110 from University of Minnesota; SEMIA 5019 from University of Hawaii, DASA31, DASA32, DASA42, DASA43, DASA44, DASA45, DASA46, DASA47, DASA48, DASA64, DASA87, DASA88, DASA89, JIRCAS 788, SMGS1, TAL99, TAL211, TAL212, TAL 213, TAL215, TAL216, TAL377, TAL379, TAL390, TAL429, TAL432, TAL778, TAL843, TAL944, TAL1906, THA1, THA2, THA3, THA5, THA6, THA7, THA9, USDA6, USDA8-0, USDA8-T, USDA35, USDA38, USDA76, USDA94, USDA117,

USDA122, USDA123, USDA136, USDA138, USDA140, USDA142, USDA143, and USDA184 from Thailand Department of Agriculture.

Rhizobium leguminosarum bv. *trifolii* strain TAL1820, *R. tropici* IFO15247 from University of Minnesota, *Rhizobium* sp. strain TAL1145 from University of Hawaii.

Sinorhizobium fredii strain HH103, *S. meliloti* strain TAL380 and TAL1372 from University of Minnesota, and *Rhizobium* sp. BL3.

Mesorhizobium ciceri strain USDA2429 and USDA2528 from University of Minnesota.

Azorhizobium caulinodans strain IRBG22, IRBG32, and DASA12005 from Department of Agriculture, Thailand.

5.3.2 Soybean seed

Soybean (*Glycine max* (L.) Merr.), Sor Jor 4 (SJ4); Sor Jor 5 (SJ5); SukhoThai 2 (ST2); Chieng Mai 2 (CM2); and Chieng Mai 60 (CM60) were obtained from the School of crop production Technology, Suranaree University of Technology.

5.3.3 Agar-plate testing for seed exudates inhibition of rhizobial growth

Rhizobial cells (10^4 cells) were inoculated onto YEM medium solidified with 0.7% (w/v) agar. Ten soybean seeds of each variety were sterilized separately by autoclaving at 121°C for 20 min (Ali and Loynachan, 1990), and were placed on the surface of inoculated agar plate (triplicate), and incubated at 28°C. The inhibition zone was observed at three or seven days depending on the rate of normal growth of

each type of rhizobia. The extent of inhibition was assessed by measuring the distance from the seed to the edge of the rhizobial growth inhibition zone (Bowen, 1961).

5.3.4 Extraction of soybean seed exudates

Seed exudates were extracted from 100 un-sterilized CM2 soybean seeds by soaking in sterile flask containing 100 ml of sterilized de-ionized water, and aerated by placing the flask on a on a rotary shaker at 200 rpm for 4 h. The seeds were rinsed with sufficient sterilized water to return the volume of extract to 100 ml. The extract was filter sterilized (0.2 μm) and 10, 30 or 50 ml of extracts were placed into sterilized tube, then frozen at -70°C for 2 h, and lyophilized over 48 h (or until completely dried). Extracts were kept in a freezer at -70°C until use (Singleton et al., 2002).

5.3.5 Testing inhibition of rhizobial growth in broth containing extracted seed exudates

Rhizobial starter cultures were grown in YEM until they reached 10^8-10^9 c.f.u./ml and washed twice with 0.9% (w/v) KCl (Stowers and Elkan, 1984). The cell pellets were diluted with YEM broth to a cell density of 10^6 cells/ml. One ml of 10^6 cells in YEM broth was placed into a tube containing the lyophilized seed exudates derived from 10, 30, 50 seeds and empty tube as the control. The cultures were mixed well prior to being placed on a rotary shaker at 28°C, 200 rpm for further growth. The growth of cells in broth containing seed exudates was determined from turbidity of the cell culture. To investigate the role of EPS on protecting cells from seed exudates, the growth of cells which were washed three times with 0.9% (w/v) KCl were compared

to non-washed cells at two different initial cell concentrations (10^6 and 10^2 cells/ml). Growth was determined by total plate count on YEM at 0, 24 and 48 h.

5.3.6 Triparental mating for transferring cloned DNA into *Rhizobium* and isolation of the clone containing genes involved in toxin tolerance

As *Sinorhizobium* sp. BL3 and *Rhizobium* sp. TAL1145 were toxin resistant and toxin sensitive strains, respectively. A genomic DNA library of BL3 was introduced into TAL1145 background by a triparental mating method (Johnston et al., 1978) using pRK2013 as a helper plasmid (Figurski and Helinski, 1979). Transconjugants were selected on YEM medium containing seed exudates derived from 25 seeds, and 10 μg/ml of Tetracycline as a selective marker. The selected cosmids were isolated by alkaline lysis method, and digested with the restriction enzymes *Eco*RI and *Hin*dIII. The plasmids used in this study are listed in Table 5.1.

5.4 Results and discussion

5.4.1 Presence of toxin in seed exudates derived from Thai soybean varieties

Five varieties of Thai soybean were used to determine the presence of toxin in seed exudates and their effects on four strains of bradyrhizobia. The distance from the seed to the edge of the rhizobial growth inhibition zone was recorded and revealed as degree of inhibition (Table 5.2). Inhibition zones were apparent for all soybean varieties with some *Bradyrhizobium* strains. The degree of inhibition varied between strains. *B. japonicum* JIRCAS788 was susceptible to seed exudates from every

soybean variety, whereas *B. japonicum* SEMIA5019 and USDA110 seemed to be resistant to seed exudates from all varieties. Variety CM2 showed the highest inhibition zone with JIRCAS788 and also found narrow inhibition zone with other strains of bradyrhizobia. Therefore, CM2 variety was selected for seed exudates extraction on next experiment.

Table 5.1 Plasmids used in this study

Plasmids	Relevant characteristics	Source or reference
pLAFR3	broad host range P1 group cloning vector, used for BL3 genomic DNA library construction, Tc^r	Staskawicz et al. (1987)
pRK2013	mobilizing cosmids, Km^r	Figurski and Helinski (1979)
pUHR301, 302, 303 and 304	pLAFR3 cosmid clone isolated from the genomic library of BL3 containing genes for toxin from seed exudates tolerance, Tc^r	This study
pUHR307	pLAFR3 cosmid clone isolated from the genomic library of BL3 containing genes for salt tolerance, Tc^r	Payakapong et al., (unpublished)

It appears seed exudates from some Thai soybean varieties inhibited the growth of bradyrhizobia, and the toxicity varied greatly between varieties. There was

an also large difference in strain sensitivity to the seed exudates. Soybean CM2 variety was bred from soybean CM60 and IAC13 varieties. CM 60 variety was bred from SJ4 and Williams soybean (DOA, www, 2005). Williams soybean seed has been reported to contain toxin in seed exudates and affects growth of *B. japonicum* TAL102 (Singleton et al., 2002). Therefore, it might be possible that soybean CM2 and CM60 derived the toxin producing genotype from Williams soybean.

Soybean seeds were sterilized by autoclaving to avoid lowering the strength of the toxin by standard methods involving rinsing with water, which the toxin may be washed away or diluted (Materon and Weaver, 1984). There have been reported that autoclaving did not influence seed toxicity and did not alter the toxin (Bowen, 1961; Masterson, 1962; Thomson, 1960). However, the high temperature of autoclaving might affect the permeability of seed coat (Ali and Loynachan, 1990). Therefore, toxin might release from the seed more than in the normal environment in which seeds germinate. However, the inhibition zone found in this experiment was narrow, revealing that Thai soybean may have very small amounts of toxic substances present in seed exudates.

5.4.2 Effect of extracted soybean seed exudates (CM2 variety) on bradyrhizobia and rhizobia growth

Soybean seed exudates were extracted from different amounts of seed and tested with bradyrhizobia and other genera of rhizobia in broth medium. In this experiment, the cells were exposed to exudates directly. The results are shown in Table 5.3. Most bradyrhizobia strains and all rhizobia grew in medium containing extracted seed exudates derived from 10 seeds. Out of 54 strains only 11 and 2 strains of bradyrhizobia that grew in medium containing extracted seed exudates derived

Table 5.2 Inhibition of bradyrhizobia by exudates from different soybean seed varieties

Rhizobial strains	Soybean varieties	Average radius of clear zone (cm)	
SEMIA5019	SJ4	0.00 ± 0.00	f
	SJ5	0.00 ± 0.00	f
	ST2	0.00 ± 0.00	f
	CM2	0.10 ± 0.00	ef
	CM60	0.00 ± 0.00	f
USDA110	SJ4	0.00 ± 0.00	f
	SJ5	0.00 ± 0.00	f
	ST2	0.00 ± 0.00	f
	CM2	0.10 ± 0.00	ef
	CM60	0.00 ± 0.00	f
JIRCAS788	SJ4	0.27 ± 0.15	cde
	SJ5	0.23 ± 0.15	cde
	ST2	0.39 ± 0.16	c
	CM2	0.91 ± 0.08	a
	CM60	0.56 ± 0.23	b
TAL213	SJ4	0.10 ± 0.00	ef
	SJ5	0.00 ± 0.00	f
	ST2	0.13 ± 0.06	def
	CM2	0.28 ± 0.17	cd
	CM60	0.00 ± 0.00	f

Means ± S.D. with different letters are significantly different at $p<0.05$

from 30 and 50 seeds, respectively. While there were no rhizobia from other genera that could grow in medium containing extracted seed exudates derived from 50 seeds, but a few strains grew in medium containing extracted seed exudates derived from 30 seeds.

Table 5.3 Growth of rhizobia from different genera in YEM medium containing extracted soybean seed exudates derived from 10, 30 and 50 seeds

Genera/strains of rhizobia	Growth of cells in medium containing extracted seed exudates		
	10 seeds	30 seeds	50 seeds
Bradyrhizobium			
DASA31	+	-	-
DASA32	+	-	-
DASA42	+	-	-
DASA43	-	-	-
DASA44	+	-	-
DASA45	+	-	-
DASA46	+	-	-
DASA47	+	-	-
DASA48	+	-	-
DASA64	+	-	-
DASA87	+	-	-
DASA88	+	-	-
DASA89	+	-	-

SEMIA5019	+	+	-
SMGS1	+	-	-
TAL99	-	-	-
TAL211	-	-	-
TAL212	+	-	-
TAL213	+	+	-
TAL215	+	-	-
TAL216	+	+	-
TAL377	+	-	-
TAL379	+	+	-
TAL390	+	-	-
TAL429	+	-	-
TAL432	+	-	-
TAL778	+	-	-
TAL843	+	-	-
TAL944	+	-	-
TAL1906	+	-	-
THA1	+	-	-
THA2	+	-	-
THA3	+	-	-
THA5	+	-	-
THA6	+	-	-
THA7	+	-	-
THA9	+	+	+

USDA6	+	-	-
USDA8-O	+	+	-
USDA8-T	-	-	-
USDA35	-	-	-
USDA38	+	-	-
USDA76	+	-	-
USDA94	-	-	-
USDA110	+	+	-
USDA117	+	-	-
USDA122	+	-	-
USDA123	+	+	-
USDA136	+	+	-
USDA138	-	-	-
USDA140	+	-	-
USDA142	+	+	-
USDA143	+	-	-
USDA184	+	+	+
Sinorhizobium			
HH103	+	-	-
BL3	+	+	-
TAL380	+	+	-
TAL1372	+	+	-
Rhizobium			
IFO15247	+	+	-

TAL1145	+	-	-
TAL1820	+	-	-
Mesorhizobium			
USDA2429	+	-	-
USDA2528	+	-	-
Azorhizobium			
DASA12005	+	+	-
IRBG23	+	+	-
IRBG32	+	+	-

+ cell could be grown in medium containing exudates

- cell could not be grown in medium containing exudates

These results reveal that the inhibition of soybean seed exudates was not specific to bradyrhizobia. The toxin from soybean seed exudates could also affect rhizobia from other genera. The non-specific effect of seed exudates has been reported in many types of leguminous seed, and also found to affect gram positive bacteria as well as gram negative bacteria (Dadarwal and Sen, 1973; Bowen, 1961). Moreover, toxicity of exudates on cells was dependant on the concentration of exudates. Most of rhizobia could tolerate exudates derived from 10 seeds, but the susceptibility was increase when increasing the concentration of exudates. Thai soybean seed exudates might not affect the cells of rhizobia under field conditions due to lower concentrations of toxin than used in the experimental design here.

5.4.3 EPS protects rhizobia from the toxic seed exudates of soybean

The effect of toxin on rhizobial growth was most apparent when EPS was removed from the cell environment before being exposed to the seed exudates. Figure 5.1 shows the effect of soybean seed exudates on the viability of cells, which were washed with KCl at different initial cell concentrations or remained in normal medium. These results indicate that the toxins present in seed exudates had a clear effect on cells without EPS regardless of the initial cell number.

Seed exudates can depress or stimulate the multiplication of organisms around seed (Bowen, 1961; Barbour et al., 1991). Exudates are known to contain a wide range of organic compounds, which could serve as a nutrient source for bacteria. This might explain the stimulation of normal rhizobial cells (without removing EPS) growing in medium amended with exudates (Fig 5.1a). The detrimental effect occurred, when EPS had been removed from the cell. Dadarwal and Sen (1973) have reported that bacteria producing large quantities of extracellular gums in the medium were less susceptible to the toxic effect of seed when compared with strains producing less gum. Moreover, the growth of polysaccharide mutants of *R. etli,* was inhibited by *Phaseolus vulgaris* root compounds (Eisenschenk et al., 1994). Therefore, EPS may be an important mechanism for protection cell from toxic compounds.

Other factors also determine the interaction between seed exudates and microorganisms in the field such as the rate of diffusion of toxic substances from seed, the concentration of toxin around seed both influenced by soil moisture and the rate of decomposition of toxin by other soil microorganisms (Bowen, 1961; Dadarwal and Sen, 1973).

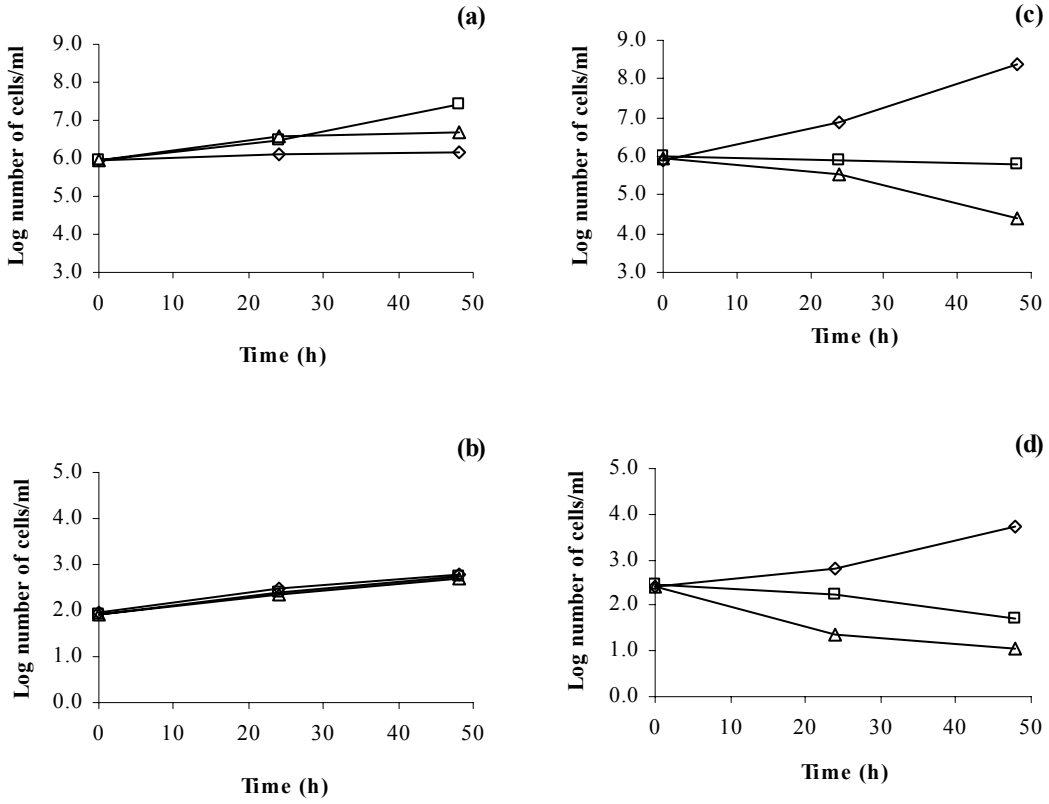

Figure 5.1 Effect of soybean seed exudates on rhizobial (TAL99) growth in broth culture (non-washed cells) at initial cell number 10^6 cells/ml (a), 10^2 cells/ml (b); and in broth culture (washed cells) at initial cell number 10^6 cells/ml (c), 10^2 cells/ml (d). Symbols were used for each parameter; YEM without exudates (open diamond), YEM containing exudates derived from 10 seeds (open square), and YEM containing exudates derived from 30 seeds (open triangle)

5.4.4 Genes involved in tolerance to toxin from seed exudates

Since toxic substances present in soybean seed exudates were non specific, fast growing rhizobia were selected to study the genes responsible for toxin tolerance. *Rhizobium* sp. TAL1145 had the toxin sensitive characteristic, whereas *Sinorhizobium*

sp. BL3 was a toxin resistant strain. BL3 could grow well in medium containing exudates derived from 10 and 30 seeds, while TAL1145 failed to grow in medium containing exudates derived from 10 seeds and tended to die when the concentration of exudates increased to 30 seeds (Fig. 5.2).

I hypothesize that *Sinorhizobium* sp. BL3 contains genes involved in tolerance to toxic substances present in soybean seed exudates. A genomic DNA library of strain BL3 was introduced into TAL1145. Transconjugants were screened for toxin tolerance by plating them on YEM agar containing extracted seed exudates derived from 25 seeds. Fourteen transconjugant colonies that grew on selected medium were selected and cosmids were isolated. Cosmids were named as pUHR301, pUHR302, pUHR303 and pUHR304. There were three common bands 3.2, 4.9 and 8.8 kb, indicating that genes involved in toxin tolerance must be located in these fragments (Fig. 5.3).

Surprisingly, one of these cosmids, pUHR301 had the same pattern of DNA band as pUHR307 after digestion with restriction enzymes. pUHR307 was a cosmid that contained genes involved in salt tolerance, which was isolated by Payakapong and colleagues (unpublished). It may be these two cosmids are identical and the mechanism for tolerance to salt stress and toxin from seed exudates might have some relationship, since autoclaved the seed may release free salts from the seed cells into the extractant. These three fragments have been sequenced by Payakapong (unpublished). Two hypothetical genes were found on 3.2 and 8.8 kb fragments. Moreover, there were two genes, which had 89% and 93% similarity to the transcriptional regulator syrB (AraC family), and xanthine dehydrogenase of *Rhizobium* sp., respectively on 4.9 kb fragment. Also another two genes, which had

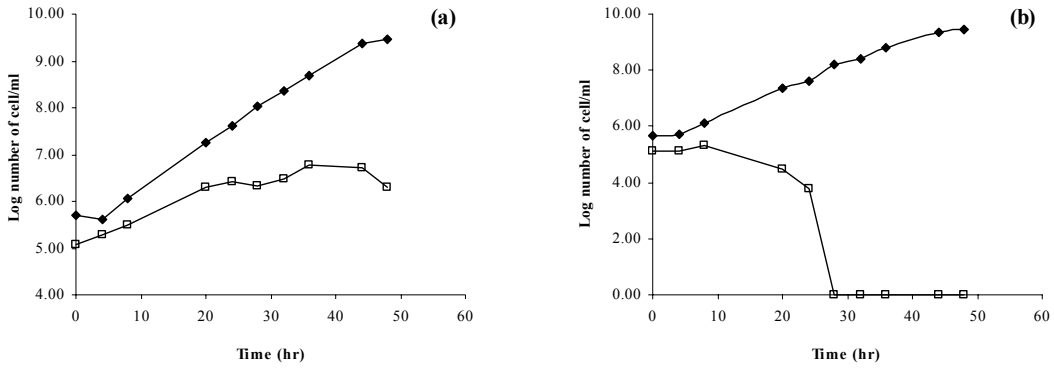

Figure 5.2 The growth of rhizobia in YEM medium containing soybean seed exudates derived from 10 seeds (a); and 30 seeds (b). Symbols were used for each strain; *Sinorhizobium* sp. BL3 (close diamond); *Rhizobium* sp. TAL1145 (open square)

67% and 89% similar to methylase gene and partitioning protein of *Arobacterium*, respectively on 8.8 kb fragment. There have been no reports on genes involved in toxin resistance to soybean seed exudates. Interestingly in the AraC family, AraC-XylS proteins are positive transcriptional regulators involved in the control of many important processes related to stress responses, pathogenesis transcriptional regulator, and carbon metabolism. BenR, a member of the AraC/XylS regulatory family, has been identified to activate the expression of genes for aromatic degradation in *Pseudomonas putida*, and involved in consumption of benzoate as carbon source (Brzostowicz et al., 2003). Many reports have identified toxic substances present in seed exudates as phenolic compounds (Fottrell et al., 1964; Thompson, 1960; Masterson, 1965; Materon and Weaver, 1984; Dhaubhadel et al., 2003). Therefore, cells that can degrade these aromatic compounds might reduce the toxicity of seed exudates. However, to confirm the hypothesis these genes should be further mutagenized and their function involving in toxin tolerance characterized.

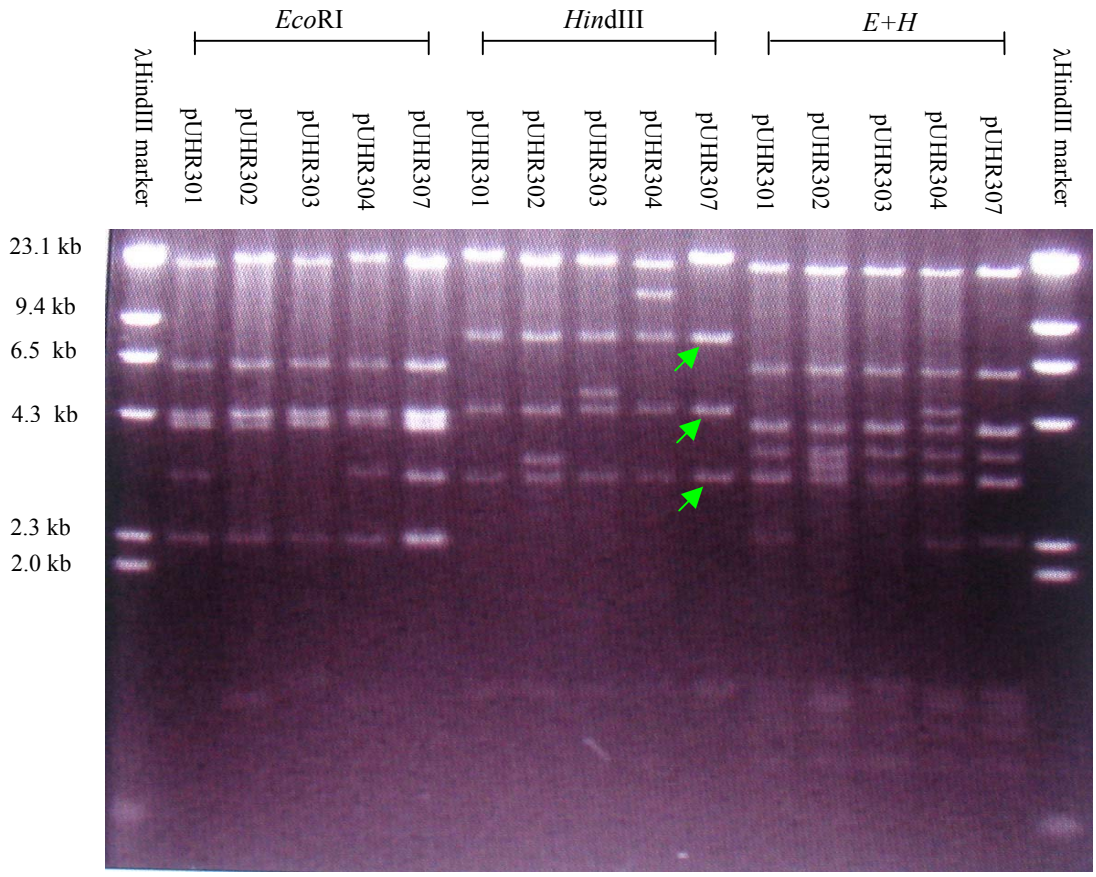

Figure 5.3 *Eco*RI, *Hin*dIII and *Eco*RI together with *Hin*dIII (E+H) digestion of cosmids isolated from TAL1145 transconjugants that grew on YEM agar containing extracted seed exudates derived from 25 seeds show four distinct types with three overlapping fragments as indicated by arrow

5.5 Conclusion

The results from this study indicate the seed of some Thai soybean varieties contain toxic substances affecting rhizobial growth. However, the degree of seed exudates toxicity varied greatly between soybean variety and bradyrhizobial strain. Seed exudates of CM2 variety showed a large effect on bradyrhizobial growth, and this effect was not specific to just bradyrhizobia. The toxin present in seed exudates inhibits the growth of rhizobia in other genera as well as *Bradyrhizobium*. The presence of EPS is an important mechanism for protecting cells from seed exudates toxins. Genetic control appears to be involved in strain tolerance to seed exudates, but it should be confirmed with further study.

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CHAPTER VI

A HISTIDENE KINASE SENSOR PROTEIN GENE IS NECESSARY FOR INDUCTION OF LOW PH TOLERANCE IN *Sinorhizobium* sp. STRAIN BL3

6.1 Abstract

Acid-sensitive rhizobial strains used as inoculant may not perform adequately in acid soils. This lack of adaptation to acid conditions may lead too poor nodulation by the inoculant strain and ultimately reduced nitrogen fixation and yield. Development of *Rhizobium* tolerant to acidic condition could improve the efficiency of inoculant. *Sinorhizobium* sp. BL3 was used as a model in this study. BL3 cannot grow at a pH lower than 6.0. To identify and isolate genes involved in adaptive acid tolerance response (ATR) from BL3, the cosmid clone library of BL3 was introduced into BL3, and the transconjugants colonies were selected at low pH. A single cosmid containing genes for ATR was isolated from forty different colonies. By transposon-insertion mutagenesis, subcloning and DNA sequencing, a gene involved in ATR named *actX* was identified in a 4.4-kb fragment of this cosmid. The *actX* mutant of BL3 showed incremental acid sensitivity and was complemented by the 4.4-kb subcloned fragment. Inoculation of *Phaseolus lathyroides* seedlings with the complemented mutant containing multiple copies of *actX* was able to increase nodulation at low pH. By constructing an *actX::gus* fusion, it was suggested that *actX*

was induced at low pH. Therefore, the *actX* gene of BL3 encoding a histidine kinase sensor protein of a two-component response regulatory system is involved in ATR.

6.2 Introduction

Soil acidity is one environmental factor that adversely affects the *Rhizobium*-legume symbiosis. Acid-sensitive rhizobial strains in inoculant may fail to nodulate properly in acidic soils poor nitrogen fixation. The development of acid tolerant rhizobia is one key to solve nodulation problems in low pH soils. Some literatures indicate that during growth on media at moderate acidity, the acid-tolerant rhizobia maintain slightly alkaline intracellular pH, whereas the acid-sensitive strains fail to maintain a neutral or alkaline intracellular pH (Chen et al., 1993; O'Hara et al., 1989). Various mechanisms, including decreasing membrane permeability, amelioration of extracellular pH, proton extrusion/uptake, and prevention of metal ion toxicity may be involved in the tolerance to low pH conditions (Dilworth and Glenn, 1999). Gram-negative bacteria, including rhizobia, are also known to have an enhanced level of tolerance to acidity if cells are grown in moderately acid condition before exposure to higher acid condition (Goodson and Rowbury, 1989; Foster and Hall, 1990; Foster, 1991). However, the mechanism for this response, known as adaptive acid tolerance (ATR) has not been identified.

At least six new proteins were induced when *Rhizobium* cells were exposed to low pH (Dilworth et al., 2001). Several genes involved in acid tolerance in *Rhizobium*, including *actA*, *actP*, *actR*, *actS*, *phrR*, and *lpiA*, have been previously identified and characterized (Tiwari et al., 1996a; Reeve et al., 2002; Tiwari et al., 1996b; Reeve et

al., 1998; Vinuesa et al., 2003). Three different approaches have been used to identify and isolate genes involved in acid tolerance or ATR. These are: (i) generation the acid-sensitive mutants from acid tolerant strains by using transposon mutagenesis, then determination the genes involved in acid tolerance (Goss et al., 1990; Tiwari et al., 1992); (ii) random insertion of a reporter gene into the rhizobial genome to create mutants in which the expression of the reporter gene is induced by low pH, then the interrupted genes are identified by sequencing (Reeve et al., 1999); and (iii) determining the differential expression of pH-dependent proteins in *Rhizobium* by one- or two-dimensional SDS-PAGE, which are then identified by N-terminal sequencing and analysis of loci encoding these proteins (Vinuesa et al., 2003).

In this study, a novel method has been developed and used to isolate a gene involved in ATR from *Sinorhizobium* sp. strain BL3, which can not grow at pH lower than 6.0. The strategy exploits the fact that acid tolerance can be enhanced by increasing the expression of certain genes involved in ATR. The derivatives of the acid-sensitive BL3 were constructed by transferring the cosmid clone library of BL3 genomic DNA into itself. Each of these derivatives contained multiple copies of a number of genes that were cloned into the cosmid. By screening these derivatives for enhanced acid tolerance, a cosmid clone containing a gene for ATR has been isolated.

6.3 Materials and methods

6.3.1 Bacterial strains and plasmids

Bacterial strains and plasmids used in this study are listed in Table 6.1.

Table 6.1 Bacterial strains and plasmids

Bacterial strains and plasmids	Relevant characteristics	Source or reference
Rhizobium strains		
BL3	*Sinorhizobium* sp., wild-type, highly Salt tolerant from Thailand, Rf^r, Sm^r	W. Payakapong and N. Boonkerd, Thailand (unpublished)
RUH175	*Act*X::Tn3 mutant of BL3, defective in growing at low pH, Rf^r, Sm^r, Km^r	This study
Escherichia coli		
DH5αMCR	Used for transformation	Bethesda Research Lab.
HB101	Use for mutagenesis, containing Tn*3*Hog*us*, Km^r	Boyer and Roulland-Dussoix (1969)
Plasmids		
pLAFR3	wide host range P1 group cloning vector, used for BL3 genomic DNA library construction, Tc^r	Staskawicz et al. (1987)
pRK404	cloning vector, Tc^r	Ditta et al. (1985)
pRK2013	used for mobilizing cosmids, Km^r	Figurski and Helinski (1979)
pPH1J1	P1 group plasmid used to eliminate pLAFR3-based cosmid from *Rhizobium* spp., Gm^r	Beringer et al. (1978)

pUC19	cloning vector, Ap^r	Beringer et al. (1978)
pUHR324	pLAFR3 cosmid clone isolated from the genomic library of BL3 containing genes for inducing acid tolerance, Tc^r	This study
pUHR325	pUHR324::Tn*3*Ho*gus*-13	This study
pUHR326	pRK404 with the 4.5 kb *Hind*III fragment carry *act*X (direction 1)	This study
pUHR327	pRK404 with the 4.5 kb *Hind*III fragment carry *act*X (direction 2)	This study

6.3.2 Media and growth conditions

Sinorhizobium strains were grown in Yeast mannitol (YEM) medium (Somasegaran and Hoben, 1994) at 28°C. *Escherichia coli* strains were grown in Luria Bertani (LB) medium at 37°C. For verifying the ATR in *Sinorhizobium* sp. BL3, cells were grown in YEM medium buffered with 20 mM HEPES (O'Hara and Glen, 1994). For internal pH determination, *Sinorhizobium* strains were grown in modified minimal salts medium (Howieson, 1985), containing (mg/l) Na_2SO_4, 100; $MgSO_4.7H_2O$, 200; $CaCl_2.2H_2O$, 5; $MnSO_4.4H_2O$, 1.11; K_2HPO_4, 4.35; KH_2PO_4, 3.4; $FeSO_4.7H_2O$, 5; $ZnSO_4.7H_2O$, 1; $Na_2MoO_4.2H_2O$, 1; $CuSO_4.5H_2O$, 0.5; thiamine HCl, 1; pantothenic acid, 1; and biotin, 0.002. For growth medium, carbon and nitrogen sources were added (g/l) arabinose, 2; galactose, 2; and glutamate, 0.4. The medium was buffered with 30 mM MES for pH values between 5.5-6.0 or 5.46 mM HEPES for the pH values between 6.5-7.0. Media were supplemented with antibiotics at final

concentrations (μg/ ml); ampicillin (Ap) 100; chloramphenicol (Cn) 30; gentamycin (Gm) 10; kanarmycin (Km) 25; rifampicin (Rf) 40; streptomycin (Sm) 100; and tetracycline (Tc) 10. The pH of media was adjusted to 5.0, 5.5, 6.0, 6.8 and 8.0 after autoclaving.

6.3.3 Determination of ATR in *Sinorhizobium* sp. BL3 and RUH175

To verify ATR effect, the cells of BL3 and RUH175 which have been grown sequentially in YEM at pH 6.5, 6.0, then finally at 5.5, and the cell of BL3 and RUH175, which have been cultured only in YEM medium at pH 7.0, were inoculated into YEM medium at pH 5.0. The growth of these two cell cultures at pH 5.0 were compared by determining the optical density of cultures at 600 nm with a spectrophotometer (Spectronic 20 Genesys^{TM}, Spectronic Instruments Inc., Rochester, NY, USA).

6.3.4 Triparental mating for transfering cloned DNA into *Sinorhizobium*

The genomic DNA library of BL3 was introduced into a BL3 background by a triparental mating method (Johnston et al., 1978) using pRK2013 as a helper plasmid (Figurski and Helinski, 1979), and transconjugants were selected on YEM medium containing Tc as a selective marker, at pH 5.0. The triparental mating method was also used to transfer plasmid DNA from *E. coli* into the acid-sensitive mutant for complementation.

6.3.5 Isolation of Tn*3*Ho*gus* insertion mutants of acid tolerant transconjugant of BL3

Cosmid pUHR324 was mutagenized by random insertion of Tn*3*Ho*gus* (a transposon constructed in the laboratory of Brian Staskawicz, University of

California, Berkeley, USA) and was also used for making *gus*-fusion mutants (Brian Staskawicz, personal communication). The mutagenization was carried out by transformation of pUHR324 into *E. coli* HB101 harboring Tn*3*Ho*gus*. The transformants were selected on LB medium containing Tc and Km. All transformants were collected and their cosmids were isolated by the alkaline-lysis method (Maniatis et al., 1982), then transformed to *E. coli* DH5αMCR for preliminary verification of the insertion point of Tn*3*, and then the inserted cosmids were selected (Borthakur et al., 2003). The derivatives of cosmid pUHR324 were transferred to wild-type BL3, and the transconjugants were spotted on YEM agar with four levels of pH (5.0, 5.5, 6.0 and 6.8). The YEM agar also contained 5 mM of 5-bromo-4-chloro-3-indolyl-D-glucuronide (X-glc), which is cleaved into a blue product by ß-glucuronidase (*gus*). Colonies were incubated at 28°C for 3-7 days. Colonies that show high expression of *gus* activity on YEM agar medium at pH 5.0, but showed low expression at neutral pH were selected. The disrupted gene in pUHR324::Tn*3*Ho*gus* of the selected derivative was transferred into the BL3 chromosome by marker exchange using the incompatible plasmid, pPH1J1 (Beringer et al., 1978), and selecting for Km and Gm resistance, but Tc sensitivity.

6.3.6 Restriction mapping of insert DNA and sequencing

A restriction map of insert DNA in cosmid pUHR324 was developed by subcloning the various *Hin*dIII and *Bam*HI fragments in pUC19, analyzing the cloned fragments by restriction digests with both restriction enzymes, and sequencing the cloned fragments from both ends using M13 forward and reverse primers by Automated DNA sequencer (Model 373A; Applied Biosystems, Foster

City, CA, USA) at the University of Hawaii Molecular Biology and Biotechnology Facilities. The position of Tn*3*Ho*gus* insertion in DNA was determined by sequencing the PCR fragment generated by using *gus* reverse primer (5' AATTCCACAGTTTTCGCGATC 3') and a forward primer from the subcloned fragment. DNA sequences were analyzed by using online program from The National Center for Biotechnology Information (NCBI).

6.3.7 Determination of internal pH

Internal pH of BL3, RUH175 and BL3:pUHR324 were determined by a fluorescent probe method (O'Hara et al., 1989). Cells were grown in 2 l of Erlenmeyer flasks containing 200 ml of minimal salts medium at pH 6.8 and 28°C for 5 days. Cells were harvested, washed twice with sterile minimal salts medium at the same pH of the respective growth medium. The washing medium was free of carbon and nitrogen. Washed cells were again suspended in the same minimal salts medium without carbon and nitrogen sources, buffered at pH 5.5, 6.0, 6.5 or 7.0 with 30 mM MES for pHs between 5.5-6.0 or 5.46 mM HEPES for pHs between 6.5-7.0. All suspensions were then incubated for 30 min at 28°C with 100 µM fluorescein diacetate (Sigma). Then cells were thoroughly washed and resuspended in the same buffer of each pH.

The fluorescence intensity of the samples was recorded with a fluorescence spectrophotometer (model F-2500; Hitachi) at 520 nm after excitation at 490 and 435 nm. The values of internal pH were read from a calibration curve prepared as described by Slavik (1982).

6.3.8 β-Glucuronidase (GUS) activity assay

The cells from 5 ml of a 12 h old culture of BL3, BL3: pUHR325 and RUH175 in YEM broth at pH 6.8, were centrifuged and resuspended in 5 ml YEM broth at pH 5.0, 6.8 or 8.0, and then allowed to grow for 9 h. The cells were precipitated and washed with 1 M NaCl, and resuspended in GUS extraction buffer containing 50 mM $NaPO_4$ (pH 7.0), 10 mM β-mercaptoethanol, 10 mM EDTA, 0.1% sodium lauryl sarcosine, and Triton X-100. The GUS activities of the samples were determined using MUG (4-methylumbelliferl-β-D-glucuronide) as the substrate and measuring the fluorescence emitted by the product 7-hydroxy 4 methylcoumarin (MU) with a 1420 Multilabel counter, PerkinElmer fluorescence spectrophotometer as described previously (Fox and Borthakur, 2001).

6.3.9 Plant experiments

Seeds of wild *Phaseolus lathyroides* were surface-sterilized, germinated and planted in modified Leonard's jar assemblies containing vermiculite and nitrogen-free nutrient solution (Somasegaran and Hoben, 1994), pH of the nutrient solution was adjusted to 5.5 or 7.0. Three-day-old seedlings were inoculated with 10^8 cells/ml of sinorhizobia BL3, RUH175, RUH175:pUHR326 and RUH175:pUHR327 from a two-day-old culture. Three replicates were used for each treatment with each jar containing three seedlings. Plants were placed in a light controlled growth chamber in a completely randomized block design and grown for 8 weeks. Nodulation was assessed by measuring the number of nodules, nodule dry weight and plant dry weight. The data were analyzed statistically using Statistical Analysis System (SAS, Computer Program, 1995). Analysis of Variance (ANOVA) and means comparison by Duncan's Multiple Range Test (DMRT) were applied to the data.

6.4 Results and discussion

6.4.1 Adaptive acid tolerance response (ATR) in BL3

Gram-negative bacteria including rhizobia, are also known to have an enhanced level of tolerance to acidity when cells are grown in moderately acid condition before exposure to higher acid condition (Goodson and Rowbury, 1989; Foster and Hall, 1990; Foster, 1991). This effect is known as ATR and also found in *Sinorhizobium* sp. BL3. The BL3 cells, which were grown in medium at pH 5.5 grew in medium at pH 5.0 faster than the cells which previously grown in medium at pH 7.0 (Fig 6.1). This result confirmed ATR in BL3 since cells which were previously exposed to a moderate acid environment adapted faster to low pH medium compared to the cells, which had not been exposed to an acid environment. This effect is also found in *E. coli* and *Salmonella* as described by O'Hara and Glenn (1994), and Foster (1991).

6.4.2 Isolation of acid tolerant derivatives of strain BL3

Genes involved in ATR were isolated by introducing a genomic DNA library of strain BL3 into strain BL3 which has a low natural level of acid tolerance (grows slowly at pH $\leq$6.0). Transconjugants were screened for acid tolerance by plating on YEM agar at pH 5.0. Forty transconjugant colonies that grew at pH 5.0 were selected. Cosmids isolated from all these transconjugants were identical (Fig. 6.2). When one of these cosmids was reintroduced into BL3, the transcojugants could grow on YEM agar at pH 5.0. Thus this cosmid named as pUHR324, enhanced the acid tolerance of BL3, indicating that it may contain gene(s) involved in ATR.

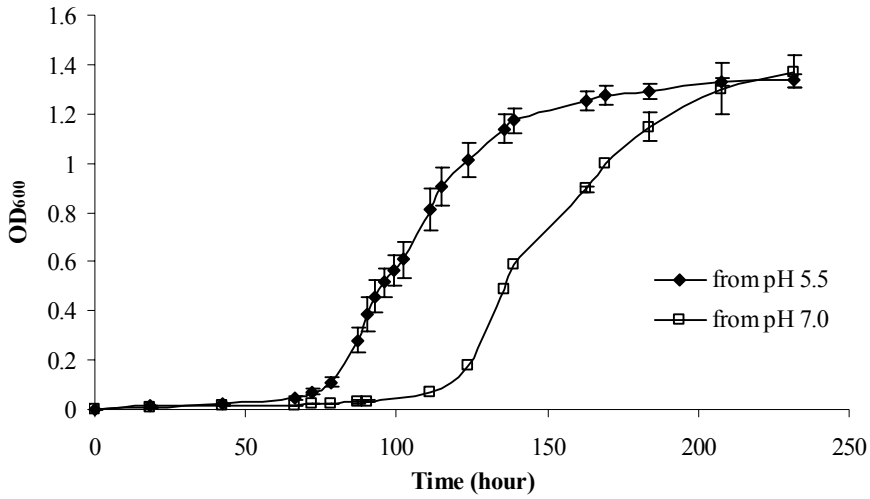

Figure 6.1 The growth of BL3 cells in YEM medium at pH 5.0 showing the ATR effect of cells, which had been transferred from medium at pH 5.5, and the cells which were transferred from medium at neutral pH 7.0

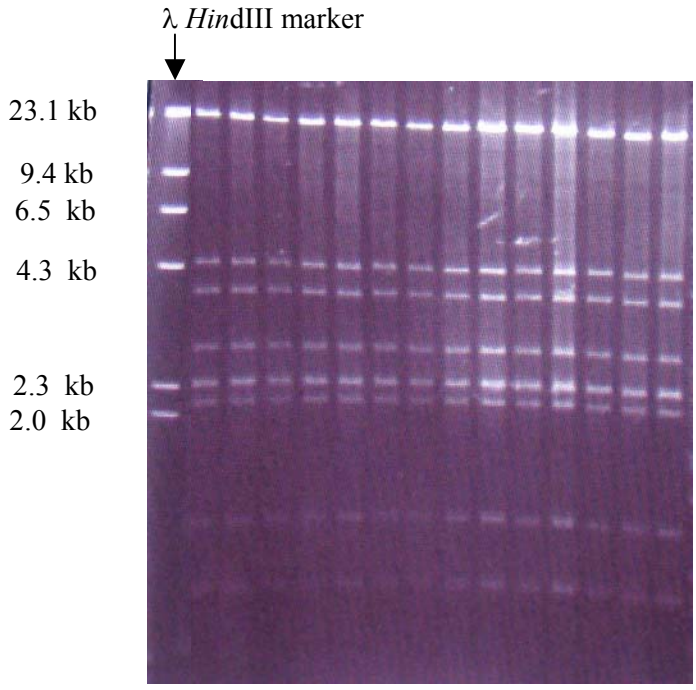

Figure 6.2 *Hin*dIII digestion of cosmids isolated from BL3 transconjugants and grown on YEM agar at pH 5.0 showing identical cosmids (named pUHR324)

6.4.3 Isolation of a gene involved in ATR from plasmid pUHR324

To identify gene(s) involved in ATR, plasmid pUHR324 was mutagenized with random insertion of the transposon Tn*3*Ho*gus*. Various pUHR324::Tn*3*Ho*gus* derivatives were transferred to BL3, and screened for colonies that showed reduced growth rate at pH 5.0. One pUHR324::Tn*3*Ho*gus* derivative (pUHR325) in BL3 that showed reduced growth rate on YEM agar at pH 5.0, but not at pH 6.8, was selected (Fig. 6.3). A mutant, RUH175, was constructed by transferring the Tn*3*Ho*gus* insertion from pUHR325 into the chromosome of BL3 by marker exchange. RUH175 grew slowly on YEM agar at pH 5.0 or 5.5, without forming single colonies. However, it grew well forming single colonies on YEM agar at pH 6.0 or 6.8. In YEM broth at pH 5.5, it showed slower growth rate than BL3 and BL3:pUHR324 (Fig. 6.4). RUH175 also showed reduced ATR when compared with the wild-type (Fig. 6.5).

Comparison of the restriction fragments of plasmids pUHR324 and pUHR325 suggested that a 4.4-kb *Hin*dIII fragment of pUHR324 was replaced by two larger fragments (4.9 and 6.2-kb) in plasmid pUHR325, indicating that the Tn*3*Ho*gus* insertion was located in the 4.4-kb fragment. Similar comparison of the *Bam*HI-digested fragments of these two plasmids indicated that the Tn*3*Ho*gus* transposon was inserted in a 1.3-kb *Bam*HI fragment (Fig. 6.6). The 4.4-kb *Hin*dIII fragment of pUHR324 was subcloned into the broad host-range plasmid vector pRK404 in both directions to obtain plasmids pUHR326 and pUHR327.

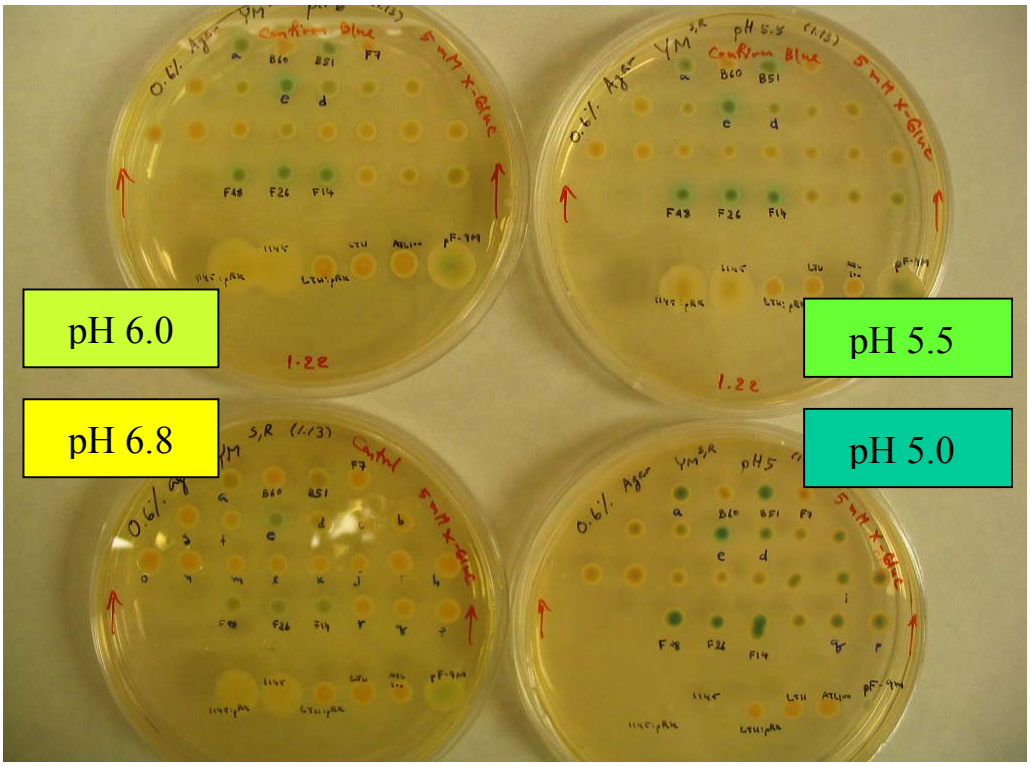

Figure 6.3 Expression of *gus*-fusion derivertives of pUHR324 in BL3 on YEM agar medium containing 5 mM of 5-bromo-4-chloro-3-indolyl-D-glucuronide (X-glc), at different pH

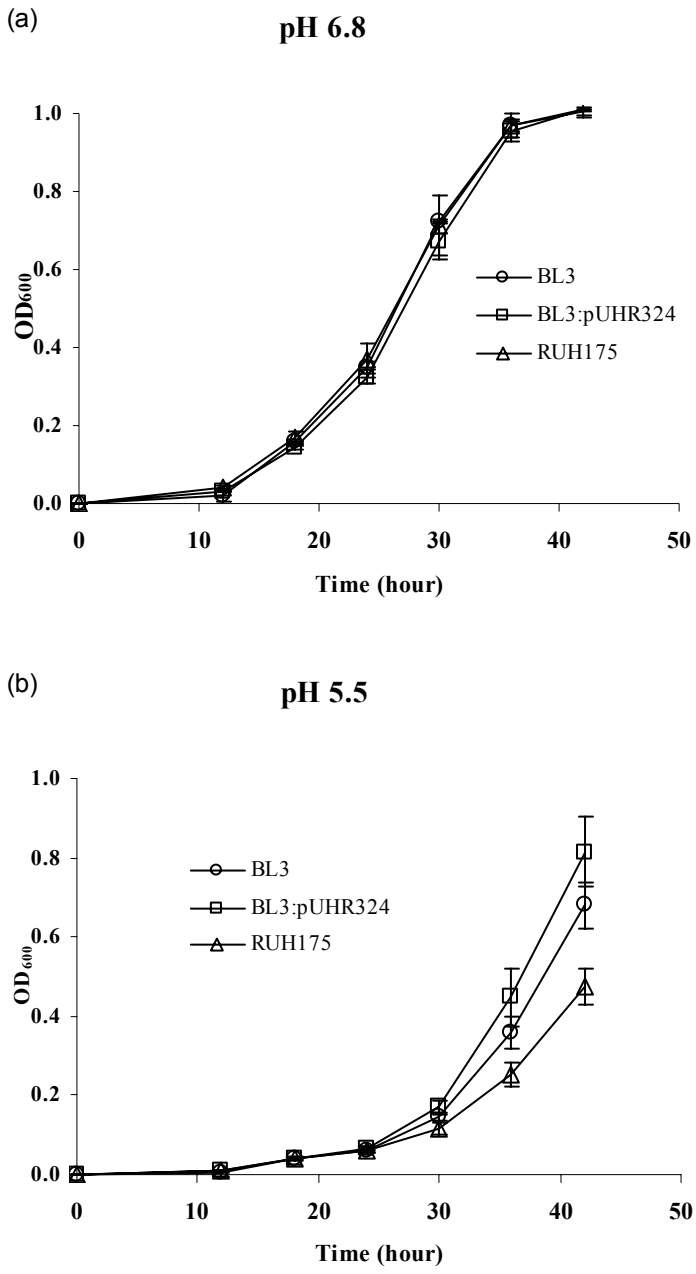

Figure 6.4 Growth of sinorhizobia in YEM liquid medium at pH 6.8 (a), and 5.0 (b). Symbols were used for BL3 (circle), BL3:pUHR324 (square) and RUH175 (triangle)

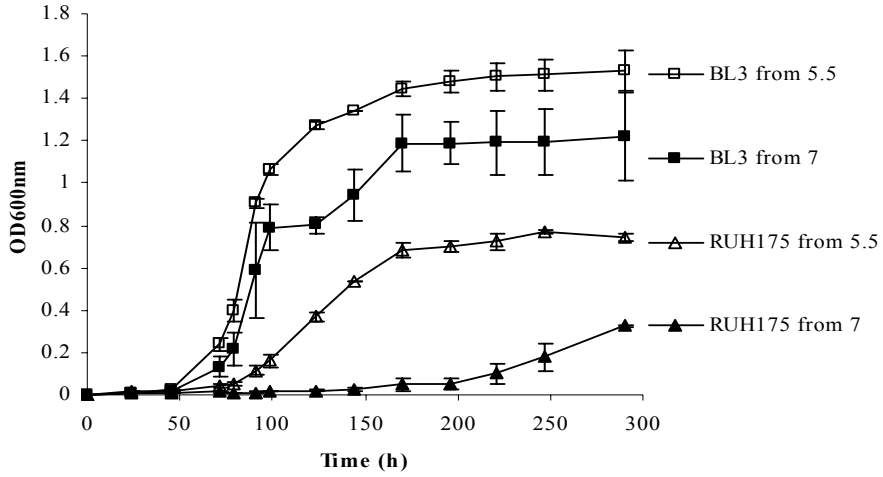

Figure 6.5 The growth of BL3 and mutant RUH175 in YEM medium at pH 5.0 showing the ATR effect of cells, which were transferred from medium at pH 5.5, and cells which were transferred from medium at pH 7.0

Restriction mapping of these plasmids suggested that they contain a 1.3-kb *Bam*HI fragment within the 4.4-kb *Hin*dIII fragment (Fig. 6.6). Both of these plasmids could complement RUH175 for the ATR defect. This 4.4-kb *Hin*dIII fragment was subcloned in pUC19 and sequenced. Sequence analyses of the 4.4-kb fragment revealed four ORFs between nucleotide positions 119 and 4,225 (Fig. 6.7). The ORF1 shows high homologies cytochrome b of *Burkholderia cepacia* and *Rhodobacter sphaeroides*. The ORF2 and ORF3 encoded proteins show high homologies with a response regulator and a histidine kinase sensor protein, respectively, of a two-component regulatory system from many bacteria including *S. meliloti*. The 1146-bp ORF4 encodes a protein of size 40.98 kDa that has up to 68% identity with CBS-domain containing membrane proteins of *Agrobacterium tumefaciens* and some other bacteria.

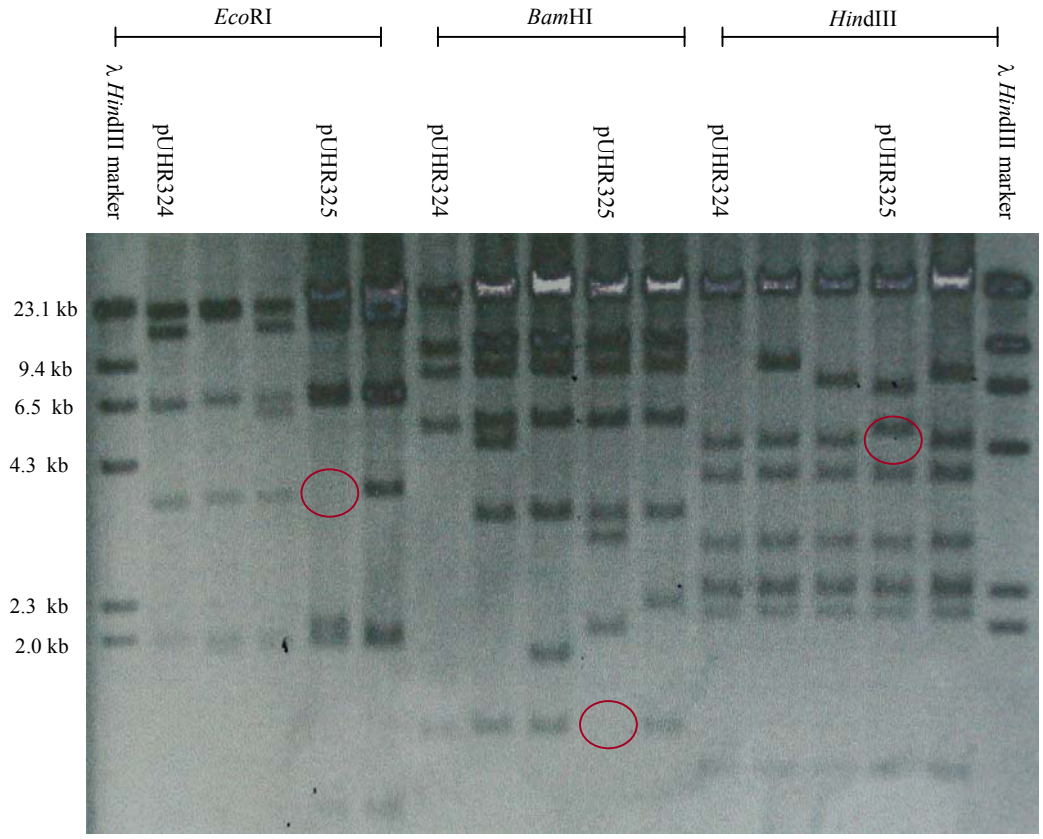

Figure 6.6 *Eco*RI, *Bam*HI and *Hin*dIII digestion of cosmid pUHR324 compared with inserted cosmid pUHR325. The Tn*3*Hog*us* transposon insertion fragments are circled

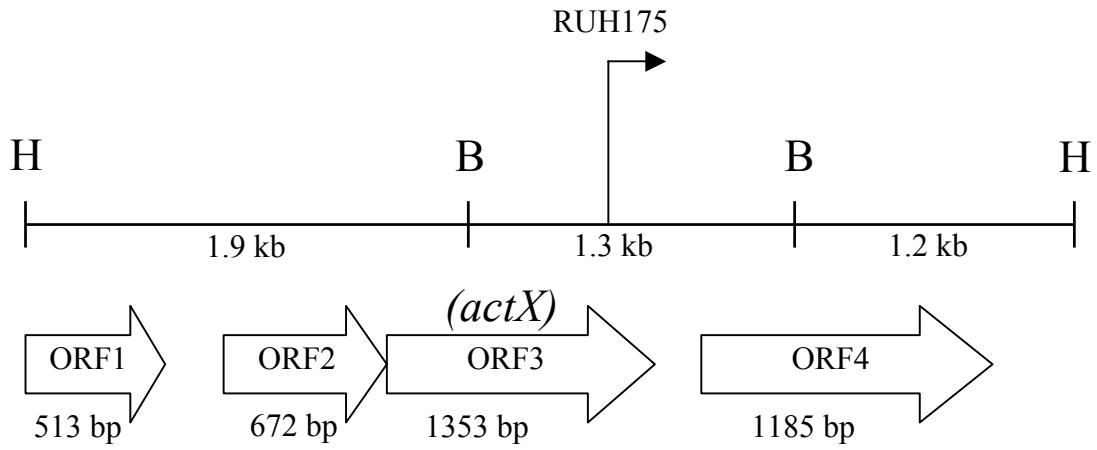

Figure 6.7 Restriction map of the 4.4-kb *Hin*dIII fragment of pUHR324 showing the position and the direction of four ORFs (open arrows). The vertical arrow indicates the position of the Tn3Ho*gus* insertion in ORF3 with the attached side arrow showing the direction of the *gus* gene in Tn*3*Ho*gus*. B: *Bam*HI, H: *Hin*dIII

The ORF3 is located between nucleotide position 1523 and 2873, and is preceded by a putative Shine-Dalgarno sequence AAGAA, 7 bp upstream of the ATG start codon. The Tn*3*Ho*gus* insertion in plasmid pUHR325 or mutant RUH175 is located within this ORF, 1250-bp downstream from the start codon. Therefore, ORF3 encodes a gene involved in ATR. Since four *Rhizobium* genes involved in acid tolerance have been previously named as *actA, actR*, *actS* and *actP*, we name this gene involved in ATR as *actX*, following the similar nomenclature. The encoded protein has a predicted molecular mass of 49.4 kDa with predicted pI of 6.2. The deduced amino acid sequence of *actX* shows 75% and 30% identities with putative histidine kinase sensor proteins of a two-component regulatory system in *S. meliloti* and *Mesorhizobium loti*, respectively. It shows 25-31% identities with two-component

sensor proteins from various Gram-negative bacteria including *Burkholderia cepacia* (31%), *E. coli* (27%), and *A. tumefaciens* (25%). ActX has all the characteristic features of a histidine kinase sensor protein of a two component regulatory system. It has a single hydrophobic transmembrane domain at position 172-192 and a conserved histidine residue (site of autophosphorylation) at postion 257. A conserved Asn at position 363 is located 106 residues apart from the conserved His-257. There is a conserved Asp-X-Gly-X-Gly domain at position 388-392, twenty-four residues downstream of Asn-363, followed by another Gly-X-Gly domain at position 413-415.

Since *actX* gene encodes a histidine kinase sensor protein of a two-component regulatory system. Previous studies by Tiwari et al. (1996b) have identified two *S. melilototi* genes, *actS* and *actR*, involved in acid tolerance. They have shown that *actS* and *actR* encode the sensor and the response regulator, respectively of a two component regulatory system. However, *actX* identified in this study does not have homology with *actS*. On the other hand, *actX* has 75% identity with a sensor protein of unknown function from *S. meliloti* (Fig. 6.8). The *actX*-like sensor gene in *S. meliloti* is followed by a response regulator gene, which has 80% identity with the ORF2-encoded protein in the present study. Unlike the *S. meliloti* homolog, ORF2 is located upstream of *actX*. The ORF2-encoded response regulator may also be involved in ATR. Since we have not isolated any mutant of BL3 in ORF2, we do not know if ORF2 is really involved in ATR.

```
ORF3:  1    MKEENPSLIGIVADASSRFRCSPWCFRSGVVFADYWFDDYKLSILMLQQETATLSRAITR 180
            MK  NPSLIGIVA     F       + GVVFADYWFDD +LS+LMLQ+ET TLS AI
SiHKP: 2    MKSRNPSLIGIVARRVIAFSLLAMILQIGVVFADYWFDDDELSVLMLQEETETLSTAIAT 61

ORF3:  181  HEGRLTYRPDHDLQDRYRVHPGDEGAIYVRVRTGSGSVLFSNCTTECAEHFLPLSVDVPS 360
             +G LT++P+H+L++RY    G  GAIYVRVRT SGSVLF+NC+ EC++HFLPL+V  P+
SiHKP: 62   QDGDLTFKPNHELKERYLERHGP-GAIYVRVRTASGSVLFTNCSAECSQHFLPLAVSAPT 120

ORF3:  361  FWKRVIEPGKPFSIAGGQSFDRNGMPVLVELAIIKDPNGFMYGALLHEMFDSMIVPMTLM 540
            FWKR+I PGKPFS+AGGQSF+R+G  VLVELA++KDPNGFMY  LL EMFDSMIVPMTLM
SiHKP: 121  FWKRLIAPGKPFSVAGGQSFERHGEVVLVELAVLKDPNGFMYSVLLREMFDSMIVPMTLM 180

ORF3:  541  FCLVIGATIWSIRGALKPVAMAVRAADAIDPRDSGARLPAARMPQEIERLVSAVNRLLAR 720
            FCLVIGATIWSIR ALKPVA AVRAADAIDPRD  ARLP++ MP+EIERLV+AVNRLLAR
SiHKP: 181  FCLVIGATIWSIRSALKPVAAAVRAADAIDPRDGNARLPSSHMPEEIERLVAAVNRLLAR 240

ORF3:  721  LGDLIQSQKLFSSSIAHEIRTPVAIAKMELSRIADPRARNAERDLDALTHILEQLTSLAR 900
            + DLIQ+QK+FSSSIAHEIRTPV+IAKMELSRI DPRARNAERDLDALTHILEQLTSLAR
SiHKP: 241  VADLIQAQKVFSSSIAHEIRTPVSIAKMELSRIDDPRARNAERDLDALTHILEQLTSLAR 300

ORF3:  901  ADAVDPSAYRRASLSEIGAEVAEATAPFVFDQGKSIEFIDHGTTPVTVIPGLVENMLRNL 1080
            ADAVDP+A++RA LS IGAEVA+ TAPFVFD GKSIEF+DHGT PV+VI  L+ENMLRNL
SiHKP: 301  ADAVDPTAFQRADLSAIGAEVAQTTAPFVFDNGKSIEFVDHGTPPVSVIAPLIENMLRNL 360

ORF3:  1081 IENAVKHNPAGTAIIVTCGPGPQVTVEDNGRGLVDLPEHNEDLGYVKRSGELGLGLKIVH 1260
            IENAVKHNP  T I++TCGPGP V+VED+GRGLVDLPEHN+DLGYVKRSG+LGLGLKIVH
SiHKP: 361  IENAVKHNPPNTCIVLTCGPGPLVSVEDDGRGLVDLPEHNDDLGYVKRSGQLGLGLKIVH 420

ORF3:  1261 RIAELHKAKIAIDTARDRGTKITIAFTAS 1347
            RI ELHKA+IA+DT    GTKITI F ++
SiHKP: 421  RIGELHKARIAVDTVPGGGTKITIDFASA 449
```

Figure 6.8 Amino acid alignment of histidine kinase sensor protein in of *S. meliloti* and inserted fragment (ORF3) in BL3

6.4.4 Intracellular pH of BL3, *actX* mutant, and BL3:pUHR324

Since some acid-tolerant strains are known to maintain higher intracellular pH, we examined if strains BL3, RUH175 and BL3:pUHR324 can maintain higher intracellular pH under low external pH conditions. At external pH 7.0, all three strains maintained a similar internal pH of approximately 9.4. However, at pH 5.5, 6.0, or 6.5, mutant RUH175, maintained significantly lower pH than BL3, and BL3:pUHR324 (Fig. 6.9). The internal pH of BL3 and BL3:pUHR324 did not show a consistent pattern of differences at different external pH conditions. These results indicate that with disruption of *actX* in mutant RUH175 the cell is unable to maintain intracellular pH at the same level as BL3 at low external pH conditions. The presence of multiple copies of *actX* in BL3:pUHR324 did not increase its intracellular pH to a level higher than that of BL3. These results suggest that the ATR of BL3:pUHR324 may involve additional mechanisms besides maintenance of high intracellular pH under low external pH conditions.

6.4.5 The *actX* gene is inducible at low pH

To determine if *actX* is induced at low pH, the *actX* mutant RUH175 with a single copy *actX::gus* fusion in the chromosome and the transconjugant strain BL3:pUHR325 containing multiple copies of *actX::gus* fusion in a plasmid, were tested for Gus activity at pH 5.0, 6.8 and 8.0 (Table 2). Both strains showed the highest level of Gus activities at pH 5.0 and slightly higher levels of Gus activities at pH 8.0 compared to pH 6.8. RUH175 had 13 folds higher Gus activities at pH 5.0 compared to pH 6.8. Similarly, BL3:pUHR325 had about 300-fold higher levels of Gus activity at pH 5.0 than at pH 6.8. These results demonstrate that *actX* is induced at acidic condition.

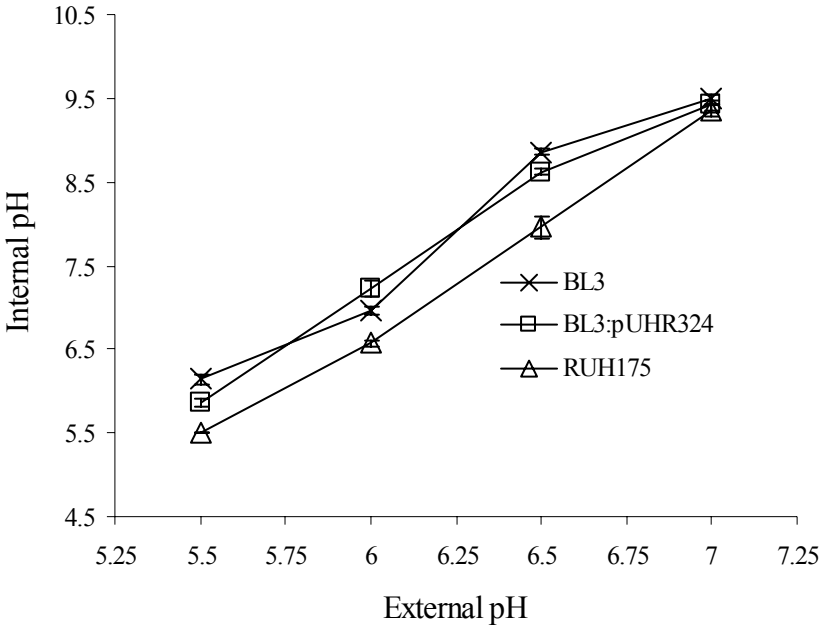

Figure 6.9 The intracellular pH of BL3 (cross), RUH175 (triangle) and BL3:pUHR324 (square) under different external pH conditions

ATR may be common in diverse bacteria including rhizobia (O'Hara and Glenn, 1994). I enhanced expression of the *actX* gene may have resulted in higher expression of certain ATR genes. ATR genes, such as *actX*, may be present in both acid-sensitive and acid-tolerant strains of rhizobia. Sensitivity or tolerance to acid may result from the level of expression of these genes. Accordingly, the acid-tolerant strains of rhizobia are expected to show high expression of both *actX* and the related response regulator gene. However, the expression of *actX* in acid-tolerant rhizobia remains to be determined.

Table 6.2 Induction of GUS activity in YEM liquid medium at different pH

Strains	GUS activity (nM of MU.ml^{-1}.h^{-1})		
	pH 5.0	pH 6.8	pH 8.0
BL3	0.0	0.0	0.0
BL3: pUHR325	4162.6 ± 89.1	14.2 ± 12.0	337.5 ± 92.5
RUH175	514.2 ± 33.8	23.6 ± 6.9	143.2 ± 107.8

6.4.6 Plant nodulation at low pH in the rooting medium

To determine the role of the *actX* gene in symbiosis, strains BL3, mutant RUH175, and the transconjugants RUH175:pUHR326 and RUH175:pUHR327 were inoculated on seedlings of *P. lathyroides*, grown in Leonard's jars in a controlled growth chamber. The *P. lathyroides* seedlings were grown at three different pH conditions: pH 5.5 and 7.0. The mutant RUH175 formed nodules as well as BL3 at all pH conditions. The number of nodules, the nodule dry weight and the plant dry weight for both RUH175 and BL3 were highest at neutral pH and decreased at lower pH conditions. At pH 7.0, strains RUH175:pUHR326 and RUH175:pUHR327 showed symbiotic performance similar to BL3. However, the plant dry weight and the number of nodules of the plants inoculated with RUH175:pUHR326 and RUH175:pUHR327 were not significantly reduced at low pH conditions, unlike plants inoculated with BL3 or RUH175. Thus the complemented mutant containing multiple copies of *actX* showed better symbiotic performance than BL3 at lower pH. These data suggested that *actX* plays an important role in symbiosis under acidic condition.

Table 6.3 Nodulation of *Rhizobium* at different pH conditions

Treatments		Plant dry wt / 1 plant (mg)		No. of nodule / 1 plant		Nodule dry wt / 1 plant (mg)	
Strain	pH						
Uninoculated	7.0	25.58 ± 9.17	c	0.00 ± 0.00	g	0.00 ± 0.00	b
	5.5	28.11 ± 0.59	c	0.00 ± 0.00	g	0.00 ± 0.00	b
BL3	7.0	41.62 ± 11.65	a	6.70 ± 0.99	bcde	4.84 ± 0.37	a
	5.5	34.40 ± 8.57	abc	4.50 ± 0.14	def	4.26 ± 0.08	a
RUH175	7.0	36.52 ± 1.17	abc	7.50 ± 1.27	b	5.16 ± 0.91	a
	5.5	29.86 ± 2.78	bc	4.37 ± 0.53	ef	4.26 ± 0.65	a
RUH175: pUHR326	7.0	41.16 ± 4.33	abc	6.62 ± 2.30	bcde	6.03 ± 0.18	a
	5.5	37.56 ± 7.51	abc	6.75 ± 0.35	bcd	4.23 ± 1.34	a
RUH175: pUHR327	7.0	40.52 ± 2.86	abc	10.08 ± 0.46	a	5.90 ± 1.81	a
	5.5	40.68 ± 2.66	abc	6.33 ± 1.89	bcdef	5.29 ± 0.77	a

Means ± S.D. with different letters are significantly different at $p<0.05$

6.5 Conclusion

An ATR gene has been identified in this work based on its ability to enhance the expression of tolerance to low pH through increasing its copy number in an acid sensitive strain. The copy number of the ATR gene was increased by transferring the cloned ATR gene in a pLAFR3-based multiple copies plasmid. The transconjugant BL3:pUHR324 showed ATR in order to grow in acid condition, although BL3 is acid sensitive. Similarly, the transconjugants RUH175:pUHR326 and RUH175:pUHR327 containing a 4.4-kb fragment cloned into pRK404 showed ATR, which may improve

the legume symbiosis in acid conditions. RUH175 derived from transposon-insertion mutagenesis of pUHR324 lost ATR capability and failed to maintain intracellular pH at low external pH. Sequencing of the DNA fragment, disrupted by the Tn*3*Ho*gus* insertion led to the identification of *actX*, which encoded a histidine kinase sensor protein of a two-component regulatory system, involved in ATR. The Gus activity due to the *actX:gus* fusion gene in mutant RUH175 and the transconjugant BL3:pUHR325 was induced at low pH condition, indicating that *actX* is indeed involved in ATR. The method of gene identification through enhanced expression using multiple copies plasmid may be used for other genes isolation which involved in tolerance to adverse environmental factors such as high temperature, high salt, and toxicity to chemicals.

6.6 References

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CHAPTER VII

THE CLONED *rpoH2* GENE OF *Sinorhizobium* sp. BL3 RESTORES EXOPOLYSACCHARIDE AND NODULATION DEFECTS OF *rpoH2* MUTANTS OF *Rhizobium* sp. TAL1145

7.1 Abstract

RpoH2 has been reported as a regulator for EPS synthesis in *Rhizobium* sp. TAL1145. To investigate the role of RpoH2 in *Sinorhizobium* sp. BL3 on EPS production, the *rpoH2* gene from BL3 was isolated by complementation of TAL1145 *rpoH2* mutant. Then mutants of the *rpoH2* gene in BL3 were generated by transposon mutagenesis. The *rpoH2* mutants of BL3 did not show significant differences for EPS production, or the pattern of small- and large-molecular weight of EPS fractionation compared to wild-type. All mutants successfully nodulated and fixed nitrogen as well as BL3. There was no direct effect of *rpoH2* on tolerance to high temperature. However, the cloned *rpoH2* gene of BL3 and *S. meliloti* restored both EPS production and nodulation defects of TAL1145 *rpoH2* mutants. Although rpoH2 of BL3 does not have a role on EPS production regulation, but the expression of this gene increased during stationary phase, therefore this gene might have the role for mediation the expression of other proteins necessary to survive in starvation conditions.

7.2 Introduction

Rhizobia in inoculants must compete with well-adapted indigenous microorganisms resident in the soil during the nodulation process. Inoculant strains also must be able to survive in the soil between crops. There are many factors affecting the persistence of rhizobial populations in the soil. These include temperature, pH, humidity, plant defense mechanisms, predators and also nutrient starvation (Brunel et al., 1988). Carrier material are not only a means to apply inoculant to seed but need to protect rhizobial cells from some of these factors as they are introduced into the soil environment. Internal mechanisms also contribute to the survival of rhizobia in soil.

It has been reported that EPS production is a survival strategy of bacteria (Ophir and Gutnick, 1994; Prakash et al., 2003). Due to its abundance, highly charged nature, and location at the outer surface of cell, EPS can protect cells against environmental stresses such as desiccation, low nutrient availability, high metallic cations, and acidity (Cunningham and Munns, 1984; Roberson and Firestone, 1992; Chenu and Roberson, 1996; Corzo et al., 1994; Spaink, 2000; Prakash et al., 2003). Moreover, EPS has a role in symbotic performance with leguminous plants. Succinoglycan or EPS I produced by *S. meliloti* has been reported as an important molecule that initiates infection of roots and elongation of infection threads by *S. meliloti* of on alfalfa roots (Cheng and Walker, 1998a; Pellock et al., 2000). Mutants of *S. meliloti*, which do not synthesize EPS or overproduce the EPS, are unable to infect alfalfa roots. However, some *exo* gene mutants induced inefficiency nodule formation (Doherty et al., 1988). EPS also is involved in the suppression of a defense

response by the host plant (Niehaus et al., 1999). Therefore, understanding the regulation of EPS production in rhizobia may elucidate its roles in supporting cell survival in adverse environments and symbiotic functioning of rhizobia.

Many factors have been reported to regulate both quantity and structure of EPS produced by *S. meliloti.* These factors include osmolarity of culture medium which regulates the relative amount of low- and high-molecular weight forms of EPS I (Breedveld et al., 1990b) and nitrogen starvation, which up-regulates EPS production and induces the production of the low molecular weight EPS I form that is involved in nodulation (Dusha et al., 1999). Also, phosphate limited conditions stimulates production of EPS II (Ruberg, 1999) while sulfur limited environments regulate EPS synthesis (Spaink, 2000). These factors may affect various genes that regulate the EPS biosynthesis in *S. meliloti*, such as *mucR*, a gene that regulates the relative level of EPS I and EPS II synthesis (Spaink et al., 1998) or other regulatory genes such as, *exoR, exsB, exoS/chvI, exoX, expG, expR, syrA* and *syrM*, which are likely to be more specific for the production of only one of the EPS classes (Cheng and Walker, 1998b; Gonz´alez et al., 1998; Dusha et al., 1999; Ruberg, 1999).

Recently, Kaufusi et al. (2004) reported the first investigation of an alternative sigma factor RpoH2 involved in the regulation of EPS synthesis in *Rhizobium* sp. strain TAL1145. The *rpoH2* mutant in TAL1145 produces only 18% of EPS produced by the TAL1145 wild-type, and show low levels of Calcofluor brightness. Inoculation of *Leucaena leucocephala* with the *rpoH2* mutant of TAL1145 resulted in produced a fewer effective nodules compared to wild-type. In *S. meliloti,* two *rpoH* genes, *rpoH1* and *rpoH2* have been identified to control expression of heat shock proteins. RpoH1 makes a minor contribution to the thermal tolerance of *S. meliloti*,

but *rpoH1* mutant affect the growth of cells at 37°C and produce poorly effective nitrogen fixing nodules on alfalfa roots. While RpoH2 is not necessary for growth at high temperature, double mutation of *rpoH1* and *rpoH2* genes prevents nodule formation (Ono et al., 2001; Oke et al., 2001). There have been no reports about the role of *rpoH2* on EPS regulation in *Sinorhizobium* species. This research examines the role of RpoH2 and the effect of *rpoH2* single mutant on EPS synthesis and symbiotic functioning of *Sinorhizobium* sp. BL3.

7.3 Materials and methods

7.3.1 Bacterial strains, plasmids and growth conditions

Bacterial strains and plasmids used in this study are listed in Table 7.1.

Table 7.1 Bacterial strains and plasmids

Strains and plasmids	Relevant characteristics	Source or reference
Rhizobia		
TAL1145	*Rhizobium* spp., wild-type strain, nodulates *Leucaena leucocephala* and *Phaseolus vulgaris*, EPS^+, Rif^r, Str^r	George et al. (1994)
RUH102, RUH103	TAL1145 *rpoH2* mutant, Km^r	Kaufusi et al. (2004)
BL3	*Sinorhizobium* sp., wild-type strain, highly salt tolerant, isolated from Thailand, Rf^r, Sm^r	W. Payakapong and N. Boonkerd, Thailand

RUH178, RUH179 and RUH180	*RpoH2*::Tn3Ho*gus* insertion mutants of BL3; Rf^r, Sm^r, Km^r	This study
Plasmids		
pLAFR3	Wide-host-range P1 group cloning vector, used for BL3 genomic DNA library construction, Tc^r	Staskawicz et al. (1987)
pRK404	Wide-host-range P1 group cloning vector, Tc^r	Ditta et al. (1985)
pRK2013	Helper plasmid used for mobilizing plasmids to *Sinorhizobium*, Km^r	Figurski and Helinski (1979)
pUC19	Cloning vector, Ap^r	Beringer et al. (1978)
pPH1J1	P1 group plasmid used to eliminate pLAFR3-based cosmid from *Rhizobium* spp., Gm^r	Maniatis et al. (1982)
pUHR323	*rpoH2* of *S. meliloti* cloned in pRK404	Kaufusi et al. (2004)
pUHR333, pUHR334, pUHR335,	Overlapping cosmid clones from the BL3 genomic library containing *rpoH2*	This study

pUHR336	PLAFR3 containing a 7.2-kb *EcoR*I fragment from pUHR333, contain *rpoH2* of BL3, Tc^r	This study
pUHR338	The 6.2-kb *Hin*dIII-*Eco*RI fragment of pUHR336 containing *rpoH2* of BL3 sucloned in pRK404, Tc^r	This study
pUHR339	The 3.1-kb segment comprising of a 0.7-kb *Bam*HI and a 2.4-kb *Bam*HI-*Eco*RI fragment from pUHR336 containing *rpoH2* of BL3 sucloned in pRK404, Tc^r	This study
pUHR340	The 2.4-kb *Bam*HI-*Eco*RI fragment from pUHR336 in pRK404, Tc^r	This study
pUHR341	The 3.0-kb *Hin*dIII-*Bam*HI fragment from pUHR336 in pRK404, Tc^r	This study

Rhizobium and *Sinorhizobium* strains were grown in yeast extract mannitol (YEM) medium (Somasegaran and Hoben, 1994) at 28°C, or at 37°C for one growth experiment. *Escherichia coli* strains were grown in Luria Bertani (LB) medium (Maniatis *et al.* 1982) at 37°C. For EPS analysis, *Sinorhizobium* strains were grown

in 10xGMS liquid medium (Breedveld et al., 1990a), and the EPS phenotype of the wild-type and mutants was also checked on 10xGMS agar medium containing 0.02% Calcofluor White M2R (Cellufluor; Polysciences). Media were supplemented with antibiotics at final concentrations of (μg/ml); ampicillin (Ap) 100; chloramphenicol (Cn) 30; gentamycin (Gm) 10; kanarmycin (Km) 25; rifampicin (Rf) 40; streptomycin (Sm) 100; and tetracycline (Tc) 10.

7.3.2 Isolation of *rpoH2* mutants of BL3 by using Tn*3*Ho*gus* insertion

Cosmid pUHR333 was mutagenized by random insertion of Tn*3*Ho*gus*, a transposon constructed in the laboratory of Brian Staskawicz, University of California, Berkeley, USA which is used to produce *gus*-fusion mutants (Brian Staskawicz, personal communication). The method for random insertion of Tn*3*Ho*gus* in cloned plasmid DNA has been described by Borthakur et al. (2003). The derivatives of cosmid pUHR333 were transferred to the *Rhizobium rpoH2* mutant, RUH102, and transconjugants that showed the colony morphology similar to RUH102 (EPS^-) were selected. The disrupted gene in pUHR333::Tn*3*Ho*gus* of the selected derivative was transferred into the BL3 chromosome by marker exchange using the incompatible plasmid, pPH1J1 (Beringer et al., 1978), and selecting for Km and Gm resistance, but Tc sensitivity.

7.3.3 Restriction mapping and sequencing of *rpoH2* gene isolated from BL3

A restriction map of the rpoH2 insertion into cosmid pUHR333 was developed by subcloning *Eco*RI fragments of pUHR333 in pLAFR3, and transferred to RUH102. The complemented subcloned plasmid was selected and subcloned in pRK404 to

obtain small fragments and transferred to RUH102. The subcloned fragment that complemented RUH102 was subcloned again in pUC19 for sequencing. The cloned fragments were sequenced from both ends using M13 forward and reverse primers by Automated DNA Sequencer (Model 373A; Applied Biosystems, Foster City, CA, USA) at the University of Hawaii Molecular Biology and Biotechnology Facilities. The position of Tn*3*Ho*gus* insertion in *rpoH2* was determined by sequencing the PCR fragment generated by using *gus* reverse primer (5' AATTCCACAGTTTTCGCGATC 3') and a forward primer from the subcloned fragment. DNA sequences were analyzed by using an online program from The National Center for Biotechnology Information (NCBI).

7.3.4 β-Glucuronidase (GUS) activity assay

The *Sinorhizobium* cells of *rpoH2* mutant, RUH180 with a single copy of the *rpoH2::gus* fusion in the chromosome and the transconjugant containing multiple copies of *rpoH2::gus* fusions in plasmid pUHR333 were grown in YEM liquid medium until late log phase. It was used to inoculate 50 ml YEM liquid medium to determine growth rate and the expression pattern of *rpoH2* gene in the cell by measuring the GUS activity at 0, 18, 24, 36, 48, 60, 72, 84, 96, 108 and 120 h. At each time point, the cells were precipitated and washed with 1 M NaCl, and resuspended in GUS extraction buffer containing 50 mM Na_2HPO_4 (pH 7.0), 10 mM *β*-mercaptoethanol, 10 mM EDTA, 0.1% sodium lauryl sarcosine, and Triton X-100. The GUS activities of the samples were determined using MUG (4-methylumbelliferl-β-D-glucuronide) as substrate and measure the fluorescence emitted by the product 7-

hydroxy 4 methylcoumarin (MU) with a 1420 Multilabel counter, PerkinElmer fluorescence spectrophotometer as described previously (Fox and Borthakur 2001).

7.3.5 EPS extraction and analyses

To determine the size fractionation of the EPS made by *Sinorhizobium* BL3 wild-type and *rpoH2* mutant RUH180, the cells were grown in 800 ml of 10xGMS liquid medium (Breedveld et al., 1990) for seven days (late stationary phase). As described by Parveen et al. (1996), cultures were centrifuged at 10,000 x g for 30 min, and the supernatant was concentrated by rotary evaporation. The concentrated supernatant was passed through 0.45 μm pore size membrane filter (Millipore Corp.), and dried by lyophilization. The dried EPS was dissolved in sodium phosphate buffer and loaded on a Bio-Gel A-5m column (39 x 4 cm). EPS were eluted by 10 mM sodium phosphate buffer pH7.0 at flow rate of approximately 0.5 ml/min. Fractions of 7 ml were collected and the hexose content determined by anthrone sulfuric acid method (Trevelyan and Harrison, 1952).

To determine the EPS production, *Rhizobium* TAL1145; *Rhizobium rpoH2* mutants RUH102, RUH103; *Rhizobium rpoH2* mutants RUH102, RUH103 containing plasmids pUHR323, pUHR339; *Sinorhizobium* BL3; *Sinorhizobium rpoH2* mutants RUH178, RUH179, RUH180; *Sinorhizobium* BL3 containing pUHR333, pUHR335 were grown in 50 ml of YEM liquid medium for seven days. One ml of culture was centrifuged at 10,000 x g for 10 min, the supernatant was collected for hexose content determination by anthrone analysis (Trevelyan and Harrison, 1952). The cell pellets were washed 3 times with 1 M NaCl, and resuspended in 1 ml of lysis buffer containing 140 mM NaCl; 2.7 mM KCl; 10 mM Na_2HPO_4; 1.8 mM KH_2PO_4; 0.1%

(v/v) Triton X-100; lysozyme at final concentration 0.2 mg/ml, and incubated at 4°C for 10 min. Samples were sonicated (amplitude 70, 6x60 sec) in ice bath. After centrifugation at 10,000 x g for 5 min, the amount of soluble protein in each supernatant phase was determined by using Lowry's method (Lowry et al., 1951).

7.3.6 Plant experiments

Sinorhizobium BL3 wild-type; BL3 transconjugants containing the plasmid cloned *rpoH2*; and their *rpoH2* mutants were inoculated on the seeds of wild *Phaseolus lathyroides* to determine the symbiosis function of *rpoH2* gene. While, *Rhizobium* TAL1145 wild-type; *Rhizobium rpoH2* mutants and *Rhizobium rpoH2* mutants containing the cloned *rpoH2* of *Sinorhizobium* BL3 and *S. meliloti* were used to inoculate *Leucaena leucocephala* and *Phaseolus vulgaris. L. leucocephala* plants were grown in modified Leonard's jar assemblies containing vermiculite and nitrogen-free nutrient solution and *P. vulgaris* was planted in sterilized sand (Somasegaran and Hoben, 1994). Three-day-old seedlings were inoculated with 10^8 rhizobia, diluted in sterile plant nutrient solution, from a two-day-old culture. Five replicates were used for each treatment with each Leonard jar containing three seedlings. Plants were placed in artificial light and organized in a completely randomized block design. P. v*ulgaris* was grown for 4 weeks, but wild *P. lathyroides* and *L. leucocephala* were grown for 8 weeks. Symbiotic functions were assessed by determining the number of nodules, nodule fresh weight, plant dry weight and nitrogenase activity. Analyses of Variance (ANOVA) and means comparison (Duncan's Multiple Range Test -DMRT) were analyzed with the Statistical Analysis System (SAS, Computer Program, 1995).

7.4 Results and discussion

7.4.1 Isolation of cosmid pUHR333 by complementing *rpoH2* mutant of *Rhizobium* sp. strain TAL1145

Less than 0.5% of the colonies in the transconjugants were mucoid (EPS^+). Fifteen of these EPS^+ colonies were purified and cosmid DNA were isolated from them. Restriction digests of these cosmids showed three distinct types with overlapping fragments. These three cosmids were transferred to both *Rhizobium rpoH2* mutant RUH102 and RUH103. They complemented both mutants. These cosmids were named pUHR333, pUHR334 and pUHR335, respectively (Fig. 7.1).

7.4.2 Localization of *rpoH2* in pUHR333 by subcloning

pUHR333 has four *Eco*RI fragments of sizes 7.0, 6.6, 6.0, and 2.2 kb. These fragments were subcloned into the *Eco*RI site of pLAFR3 and the resulting plasmids were transferred to RUH102. One of these plasmids, pUHR336 containing the 7.0-kb fragment complemented both RUH102 and RUH103. A 6.0-kb *Hin*dIII-*Eco*RI subclone from the 7.0-kb *Eco*RI fragment in plasmid pUHR338 also complemented both RUH102 and RUH103. A 3.0-kb *Hin*dIII-*Bam*HI fragment or a 2.3-kb *Bam*HI-*Eco*RI fragment did not complement these mutants. However, a 3.0-kb segment comprising of a 0.7-kb *Bam*HI and the 2.3-kb *Bam*HI-*Eco*RI fragment, cloned in plasmid pUHR339 complemented these mutants. Therefore, the *rpoH2* gene is located within this 3.0-kb *Bam*HI-*Eco*RI region (Fig. 7.2).

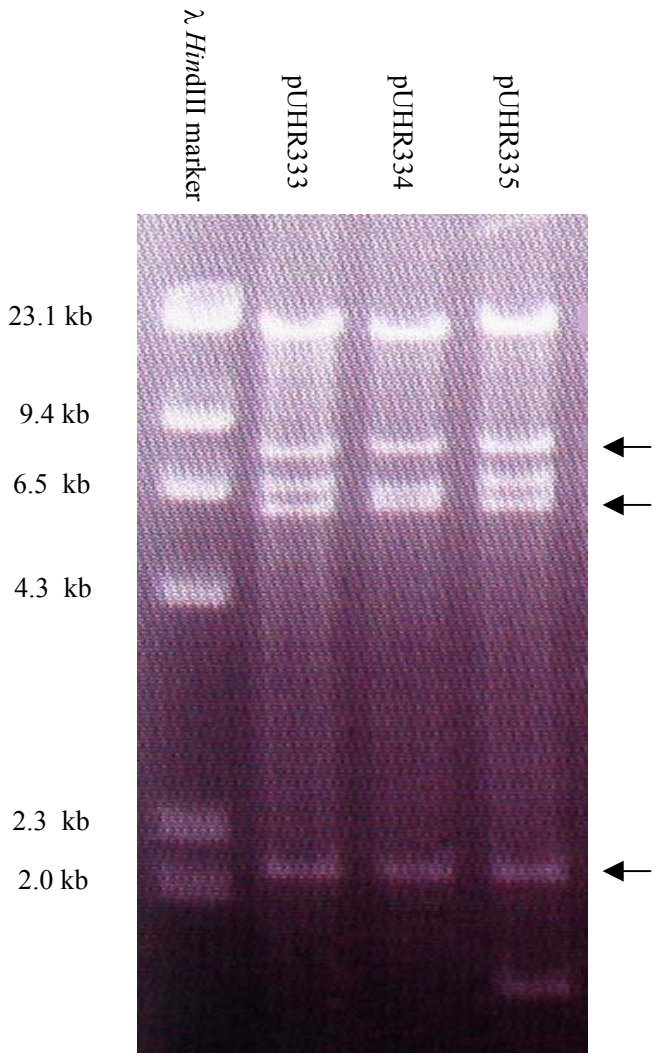

Figure 7.1 *Eco*RI digestion of *rpoH2* complemented cosmids showed three distinct types with overlapping fragments as indicated by arrow

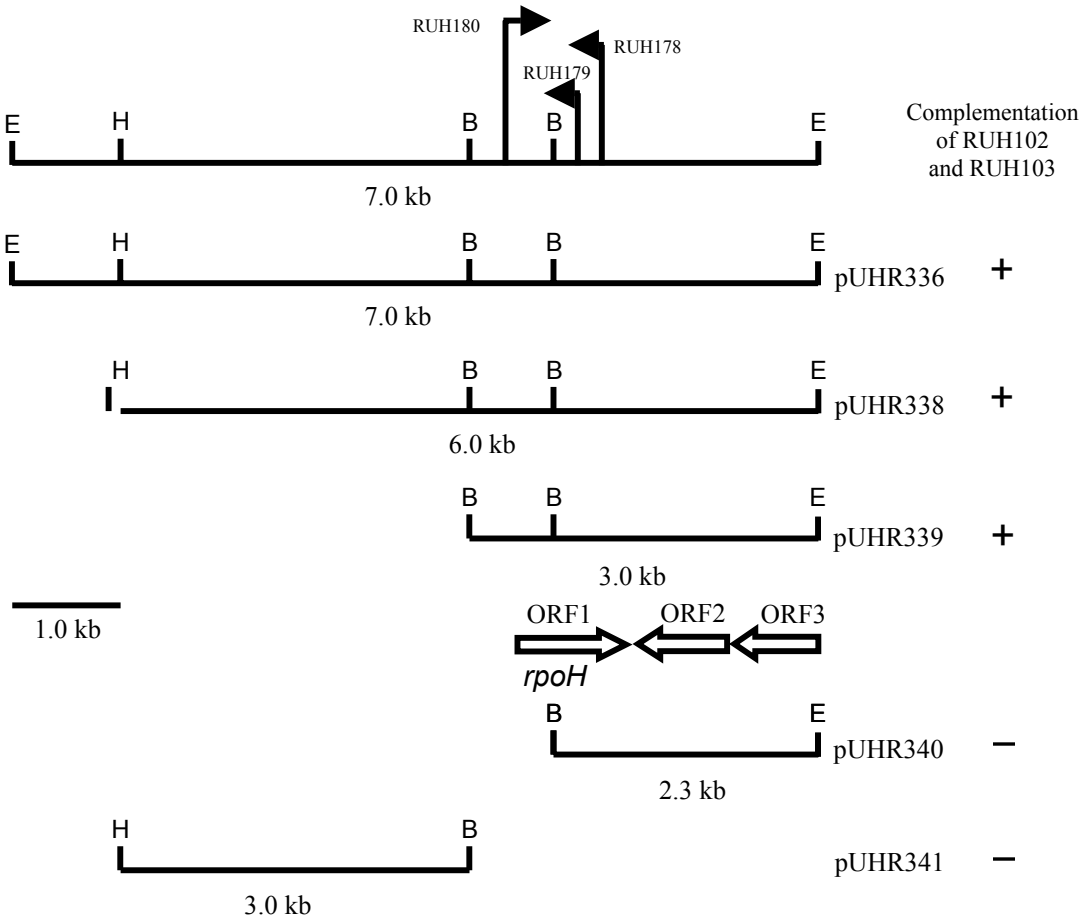

Figure 7.2 Restriction map of the 7.0-kb region subcloned from cosmid pUHR336 that contains the *rpoH2* gene from *Sinorhizobium* sp. strain BL3. Smaller subcloned fragments from the 7.0-kb region are also demonstrated. The horizontal open arrow shows three ORFs; ORF1 (*rpoH2*); ORF2 (amino acid transport system permease ABC transporter protein); ORF3 (amino acid binding periplasmic (signal peptide) ABC transporter protein). The positions of the three *ropH2*::Tn3Ho*gus* insertions in mutant RUH178, RUH179, and RUH180 are shown with solid arrows where the direction of the arrow indicates the direction of the *gus* reporter gene. B: *Bam*HI, E: *Eco*RI, H: *Hin*dIII

7.4.3 Isolation of *rpoH2* mutants of BL3

pUHR333 was mutagenized with Tn3Hog*us* insertion and the resulting pUHR333:Tn3Hog*us* derivatives were transferred to RUH102. Twenty-two colonies showing the colony morphology similar to RUH102 were selected. In these colonies, the Tn3Hog*us* insertions are expected to be inserted on the *rpoH2* gene. Plasmid DNA was isolated from these colonies, and analyzed by restriction digest. Three pUHR333::Tn3Hog*us* derivatives that showed distinguishable insertion positions, were selected (Fig. 7.2). These insertions were transferred to BL3 and the interrupted genes were marker-exchanged into equivalent positions in the chromosome. In this way three mutants were isolated. These are RUH178, RUH179, and RUH180.

7.4.4 The nucleotide sequence of *rpoH2* isolated from BL3

To verify the interrupted position of Tn3Hog*us* in *rpoH2* gene, the 3.0-kb *Bam*HI-*Eco*RI fragment of pUHR339 was sequenced. Analysis of the sequence revealed three ORFs between nucleotide positions 394 and 2916. The ORF1 encoded a protein shows high homology with sigma factor *rpoH2* of *S. meliloti*, including *rpoH*-like sigma factor of *Rhizobium* sp. TAL1145. The ORF2 and ORF3 were located in the reverse direction with ORF1, show 72% and 85% homology with putative amino acid transport system permease ABC transporter protein and putative amino acid binding periplasmic (signal peptide) ABC transporter protein of *S. meliloti* 1021, respectively.

The ORF1 is located between nucleotide positions 394 and 1251 on 3.0-kb fragment of pUHR339 (Fig. 7.3). The results of sequence analysis reveal the insertion point of Tn3*Hogus* on nucleotide positions 40; 585 and 805 of ORF1. These

nucleotides of ORF1 encode a protein of 32.707 kDa with a pI 9.5. This protein shows 82% similarity to sigma factor *rpoH2* of *S. meliloti*, 71% similarity to *rpoH*-like sigma factor of *Rhizobium* sp. TAL1145, and also shows 59% similarity to *rpoH*-like sigma factor C of *Mesorhizobium loti* MAFF303099. Therefore, the interrupted gene cloned from *Sinorhizobium* sp. BL3 can be designed as a *rpoH2*-like gene due to high similarity with *rpoH2* in *S. meliloti*.

7.4.5 Expression of *rpoH2*

To investigate the pattern of *rpoH2* gene expression in *Sinorhizobium* sp. BL3 during growth, the Gus activities of the *rpoH2* mutant RUH180 with a single copy *rpoH2::gus* fusion in the chromosome and the transconjugant containing multiple copies of *rpoH2::gus* fusion in plasmids pUHR333 and pUHR335 were determined in the background of BL3 in YEM medium. The gene was expressed during cell growth, however, expression was at a low level. Gus activities increased when the cell entered stationary phase. Then, the expression of the gene in the transconjugant containing multiple copies of *rpoH2::gus* fusion and *rpoH2* mutant RUH180 increased about 2 and 1.2 times during stationary phase, respectively (Fig. 7.4).

Due to the expression of *rpoH2* gene of BL3 was highly increased when the cell entry to the stationary phase. It is reminiscent of the RpoH function in carbon starvation. In *E. coli*, the level of RpoH protein increases during glucose starvation, and the *rpoH* mutant strain shows decrease survival during starvation (Jenkins et al., 1991). Moreover, the RpoH-dependent heat shock protein, DnaK and HtpG are nutritionally regulated and the DnaK mutant strain poorly survived in carbon starvation when compared with wild-type strain (Spence et al., 1990).

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BL3rpoH2: 394  MKTLTADRRLIKIAMDAPYLERDEEHALAEAWRYDHDQEARNKIAMSHMRLVVAMAAKFR 573
               MKTLTADRR+IKIAM+APYLERDEEHALA+AWR D+DQEARNKIAMSHMRLV++MAAKFR
1021rpoH2: 1   MKTLTADRRMIKIAMEAPYLERDEEHALAQAWRNDNDQEARNKIAMSHMRLVISMAAKFR 60

BL3rpoH2: 574  SFGLPMGDLVQEGHIGLLEAAARFEPSREVRFSTYATWWIRASMQDYVLRNWSIVRGGTS 753
               SFGLPMGDLVQEGHIGLLEAAARFEPSREVRFSTYATWWIRASMQDYVLRNWSIVRGGTS
1021rpoH2: 61  SFGLPMGDLVQEGHIGLLEAAARFEPSREVRFSTYATWWIRASMQDYVLRNWSIVRGGTS 120

BL3rpoH2: 754  SAQKALFFNLRRLRARLAQGDRQLTSQAMHEEIAAALGVSLADVQTMDARLSGNDASLQA 933
               SAQKALFFNLRRLRARLAQGDRQLTSQAMHEEIAAALGVSLADVQTMDARLSGNDASLQA
1021rpoH2: 121 SAQKALFFNLRRLRARLAQGDRQLTSQAMHEEIAAALGVSLADVQTMDARLSGNDASLQA 180

BL3rpoH2: 934  PVGHGDAESGARLDFLPSDAPLPDEQVSEMIDGERARRWLHSALRELSEREMKIIRARRL 1113
               P+G GD ++GARLDFL S+APLPDEQVS++IDGERARRWL  AL ELSEREMKIIRARRL
1021rpoH2: 181 PIGSGDPDAGARLDFLASEAPLPDEQVSDLIDGERARRWLQVALGELSEREMKIIRARRL 240

BL3rpoH2: 1114 SEDGATLEELGVALGISKERVRSKRGRSKSCVRR--SPRAPALTAGMH 1251
               +EDGATLEELGVALGISKERVR    R+   +R   + +APALTA MH
1021rpoH2: 241 TEDGATLEELGVALGISKERVRQIETRALEKLRAALTAKAPALTASMH 288
```

Figure 7.3 Protein sequence comparison between *rpoH2* gene of *Sinorhizobium* sp. BL3 and *S. meliloti* Rm1021

Thorne and Williams (1997) have reported that entry of bacterial cells, including rhizobial, into stationary phase leads to multiple stress resistances, such as protection against pH, heat, oxidants and osmotic shock. Although RpoS has been found to mediate the stress response in *E. coli* during stationary phase (Hengge-Aronis, 1996; Woësten, 1998), *rpoH* was also expressed very high during stationary phase. It may be that *rpoH* regulates some additional proteins responsible for stress resistance, which are developed during starvation.

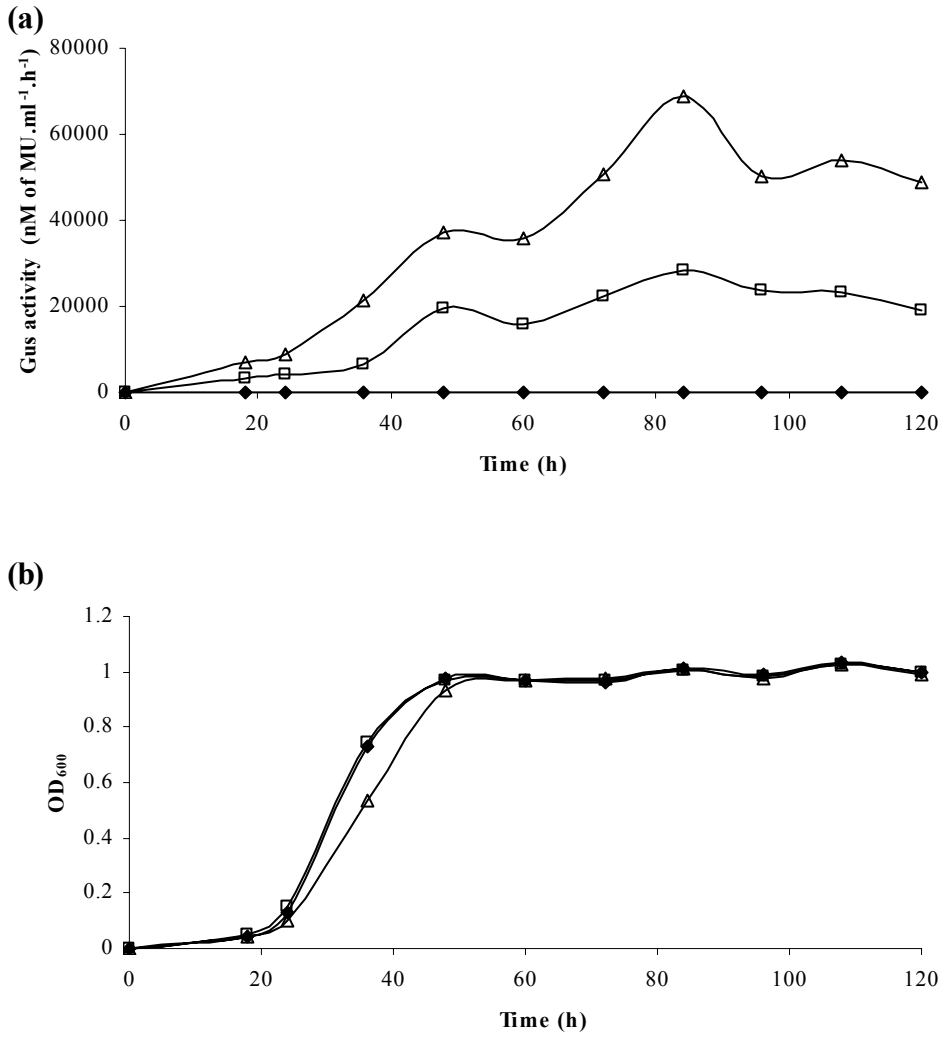

Figure 7.4 Expression of *rpoH2* represented via β-glucuronidase activity (a), and the growth (b), of wild-type BL3 (close diamond); *rpoH2* mutant RUH180 (open square); and transconjugant BL3:pUHR339 (open triangle), in YEM medium at 28°C

7.4.6 Characteristics of *rpoH2* mutants

Mutants RUH178, RUH179 and RUH180 produced almost the same amounts of EPS as BL3 (Table 7.2). The fractionation of high and low molecular weight EPS produced by the mutants showed similar patterns as the wild-type (Fig. 7.5). The EPS produced by *S. meliloti* can be divided to two different size classes, high molecular weight EPS, which consist of thousands of saccharide units, and low molecular weight EPS, which consist of 8-40 saccharide units (Spaink, 2000). Two distinct peaks, representing high and low molecular weight components, were obtained. Both components were estimated to be well in excess of 300 kDa, based on the fractionation range of Bio-Gel A-5M.

There were no significant differences in growth between the transconjugants of BL3 containing pUHR333, mutant RUH180 and wild-type when grown in YEM medium at 28°C or 37°C (Fig. 7.6). Therefore, this result supports previous papers, which reported that *rpoH2* is not necessary for thermal tolerance in *Sinorhizobium* species, even when this same gene complements the temperature sensitivity of *E. coli* strain (Ono et al., 2001; Oke et al., 2001).

Nodulation and nitrogen fixation by *rpoH2* mutants were compared with pBL3 using *P. lathyroides* as a host plant. The results demonstrated all mutants could successfully nodulate and fix nitrogen as well as wild-type (Table 7.3). Therefore, mutation in the *rpoH2* gene did not affect the EPS regulation or symbiosis function of *Sinorhizobium* sp. BL3 which is a different result than the outcome of *rpoH2* in *Rhizobium* sp. TAL1145 (Kaufusi et al., 2004).

Table 7.2 Exopolysaccharide production by *Rhizobium* TAL1145 *rpoH2* mutants containing the cloned *rpoH2* gene of *Sinorhizobium* BL3

Strains	Colony morphologies	Exopolysaccharide (mg/mg of protein)	
TAL1145	Mucoid, Cal+	10.963 ± 1.68	ab
RUH102	Non-mucoid, Cal-	0.683 ± 0.25	f
RUH103	Non-mucoid, Cal-	0.559 ± 0.08	f
RUH102:pUHR339	Mucoid, Cal+	10.336 ± 2.44	abc
RUH103:pUHR339	Mucoid, Cal+	10.314 ± 2.11	abc
RUH102:pUHR323	Mucoid, Cal+	8.986 ± 1.28	c
RUH103:pUHR323	Mucoid, Cal+	7.186 ± 1.34	d
BL3	Mucoid, Cal-	4.719 ± 0.32	e
RUH178	Mucoid, Cal-	4.139 ± 0.35	e
RUH179	Mucoid, Cal-	4.233 ± 0.61	e
RUH180	Mucoid, Cal-	3.012 ± 0.77	e
BL3:pUHR333	Mucoid, Cal-	4.423 ± 0.78	e
BL3:pUHR335	Mucoid, Cal-	4.535 ± 0.19	e

Means ± S.D. with different letters are significantly different at $p<0.05$

Cal- ; Calcofluor-binding acidic EPS of rhizobia exhibit dim under UV

Cal+ ; Calcofluor-binding acidic EPS of rhizobia exhibit blue-green fluorescence under UV

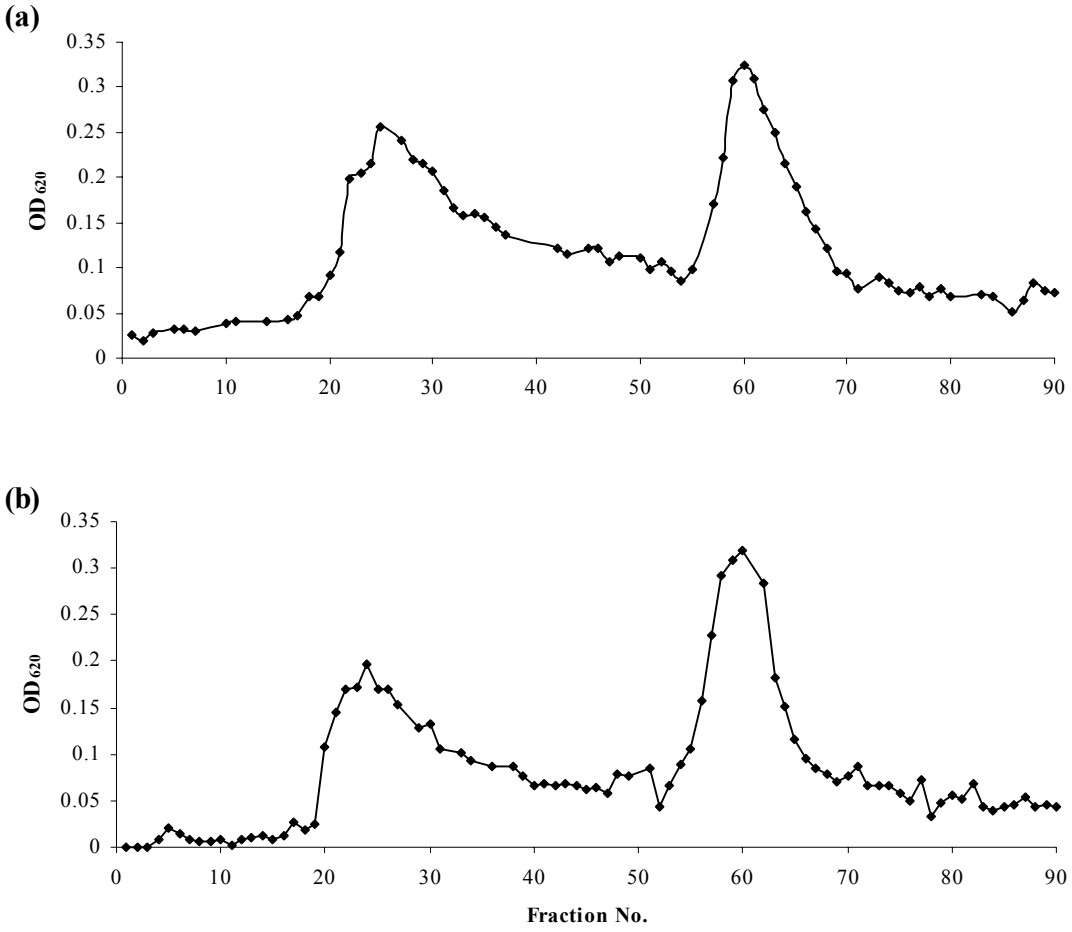

Figure 7.5 Gel filtration of the culture supernatant of (a) BL3 and (b) rpoH2 mutant (RUH180) through a Bio-Gel A-5 m column. The lyophilized culture supernatant was loaded on the column and the hexose content of the samples was determined by Anthrone sulfuric method

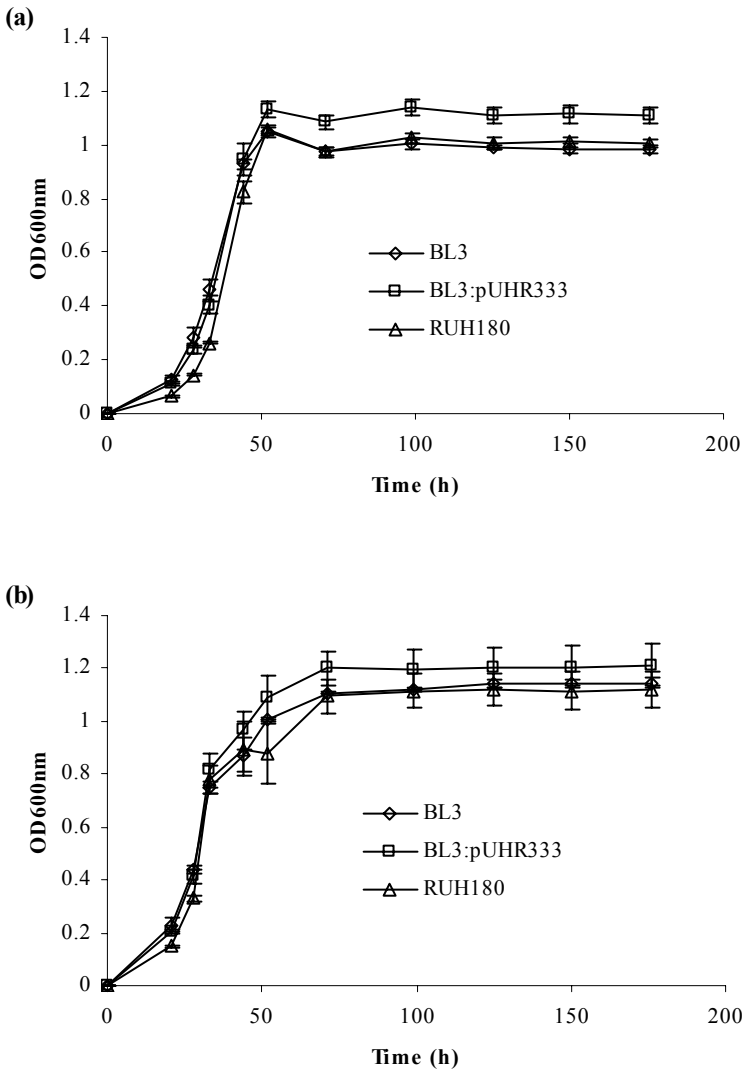

Figure 7.6 Comparison of the growth of wild-type (BL3) cells with the growth of *rpoH2* mutants (RUH180) and BL3 containing the cloned *rpoH2* plasmid (pUHR333) in YEM medium at 28°C (a), and 37°C (b)

7.4.7 Complementation of *Rhizobium* TAL1145 *rpoH2* mutants by the cloned *rpoH2* gene of *Sinorhizobium* BL3

Although *rpoH2* of *Sinorhizobium* sp. BL3 and *S. meliloti* showed 71% and 75% similarity to a *rpoH*-like sigma factor of *Rhizobium* sp. TAL1145, respectively. *rpoH2* of *Sinorhizobium* sp. BL3 and *S. meliloti* did not involve EPS production or symbiotic functions. Therefore, to determine whether the function of *rpoH2* in *Rhizobium* can be complemented by *rpoH2* gene isolated from *Sinorhizobium* the cloned *rpoH2* gene of BL3, pUHR339; and pUHR323 isolated from *S. meliloti* 1021 were introduced to TAL1145 *rpoH2* mutants, RUH102 and RUH103. The colony morphologies and the amounts of EPS produced by different transconjugants containing the cloned *rpoH2* gene of *Sinorhizobium* are shown in Table 7.2. On medium containing Calcofluor, the colony of mutants containing the cloned *rpoH2* genes of both *Sinorhizobium* BL3 and *S. meliloti* 1021 exhibit the characteristic bright color as well as the wild-type. The cloned *rpoH2* genes of *Sinorhizobium* BL3 could fully complement the EPS defect in RUH102 and RUH103, while the cloned *rpoH2* genes of *S. meliloti* 1021 could complement the EPS defect but did not produce as much EPS as TAL1145. Moreover, the transconjugants containing the cloned *rpoH2* gene of both *Sinorhizobium* BL3 and *S. meliloti* 1021 were inoculated onto seedlings of *L. leucocephala* and *P. vulgaris* to compare symbiotic function with the wild-type and *rpoH2* mutants. The results showed that the cloned *rpoH2* gene of both *Sinorhizobium* BL3 and *S. meliloti* 1021 also complemented the nodulation and nitrogen fixation defects of RUH102 and RUH103 on both *Leucaena* and *P. vulgaris* (Table 7.4 and 7.5). In *Leucaena*, a few nitrogen-fixing nodules were found on the plants inoculated with *rpoH2* mutants, while the plants inoculated with the cloned

rpoH2 gene from both *Sinorhizobium* restored symbiotic functioning of the plant as well as TAL1145. Interestingly, the *rpoH2* mutant of TAL1145 could not form nodules on the roots of kidney beans. However, there were no significant differences in nodulation and nitrogen fixation between plants inoculated with transconjugants containing *Sinorhizobium rpoH2* gene and the wild-type. Therefore, the *rpoH2* defect in *Rhizobium* can be complemented by *rpoH2* gene isolated from *Sinorhizobium*. These results confirmed the phylogenetic tree that showed very close relationship between the *rpoH2* of *Rhizobium* TAL1145 and *S. meliloti* (Kaufusi et al., 2004).

7.5 Conclusion

Kaufusi and his colleges (2004) investigated the role of RpoH2 in EPS synthesis and symbiotic functions of *Rhizobium* sp. TAL1145, and suggested that RpoH2 of TAL1145 and *Sinorhizobium meliloti* may have similar regulatory functions. However, this research demonstrates that the single mutation of *rpoH2* in *Sinorhizobium* sp. BL3 was not involved in regulation of EPS production and symbiosis with host plant. The *rpoH2* mutants in BL3 could successfully nodulate and fix nitrogen as well as wild-type. These mutants did not have any defect in EPS production. All mutants produced the same amount of EPS and also showed a similar profile of small and large molecular weight EPS fractions compared to EPS produced by the wild-type. There were no significant differences in growth between the transconjugants of BL3 containing pUHR333, mutant and wild-type when grown in medium at 28°C or 37°C. However, the cloned *rpoH2* gene of *Sinorhizobium* sp. BL3 and of *S. meliloti* restores both EPS and nodulation defects of *rpoH2* mutants of *Rhizobium* sp. TAL1145.

Table 7.3 Nodulation of *Phaseolus lathyroides* by *Sinorhizobium* BL3, BL3 containing the cosmid cloned of *rpoH2* gene of *Sinorhizobium* BL3 and *rpoH2* mutants

Strain	Nodule number / plant	Nodule fresh wt (g)/plant	Nitrogenase activity (µmol of ethylene/nodule fresh wt (g)/h)	Plant dry wt (mg)/plant
BL3	11.8 ± 2.3 a	0.061 ± 0.009 a	1.980 ± 0.465 a	280.89 ± 69.10 ab
BL3:pUHR333	7.8 ± 1.4 bc	0.086 ± 0.020 a	1.681 ± 0.661 ab	187.20 ± 47.89 c
BL3:pUHR335	12.5 ± 2.2 a	0.070 ± 0.019 a	1.221 ± 0.763 abc	299.49 ± 18.35 a
RUH178	5.4 ± 0.8 c	0.052 ± 0.010 a	1.085 ± 1.648 abc	235.56 ± 63.63 abc
RUH179	5.3 ± 2.4 c	0.037 ± 0.015 a	1.145 ± 0.811 abc	212.66 ± 64.99 bc
RUH180	10.2 ± 3.6 ab	0.076 ± 0.025 a	0.648 ± 0.712 bc	221.23 ± 49.55 bc
Uninoculated (control)	0.0 ± 0.0 d	0.000 ± 0.000 b	0.000 ± 0.000 c	38.62 ± 27.89 d

Means ± S.D. with different letters are significantly different at $p<0.05$

Table 7.4 Nodulation of *Leucaena leucocephala* by *Rhizobium* TAL1145 *rpoH2* mutants containing the cloned *rpoH2* gene of *Sinorhizobium* BL3 and *Sinorhizobium meliloti* 1021

Strain	Nodule number / plant	Nodule fresh wt (g)/plant	Nitrogenase activity (µmol of ethylene/nodule fresh wt (g)/h)	Plant dry wt (mg)/plant
TAL1145	10.6 ± 1.7 a	0.000 ± 0.000 ab	13.55 ± 2.97 bc	185.57 ± 10.87 a
RUH102	3.2 ± 1.8 d	0.019 ± 0.006 def	6.97 ± 0.70 def	117.55 ± 8.22 d
RUH103	3.2 ± 0.5 d	0.026 ± 0.007 cde	6.44 ± 2.65 ef	163.44 ± 7.47 b
RUH102:pUHR336	6.6 ± 2.3 bc	0.034 ± 0.018 bcd	12.21 ± 6.10 bcd	139.55 ±8.12 c
RUH103:pUHR336	8.9 ± 1.2 ab	0.048 ± 0.017 ab	16.72 ± 4.89 ab	161.25 ± 12.82 b
RUH102:pUHR339	7.0 ± 1.7 bc	0.062 ± 0.021 a	10.37 ± 2.88 cde	178.25 ± 13.71 a
RUH103:pUHR339	6.8 ± 1.0 bc	0.045 ± 0.004 ab	13.06 ±3.95 bc	138.95 ± 3.11 c
RUH102:pUHR323	10.7 ± 2.8 a	0.041 ± 0.007 bc	12.72 ± 2.74 bc	131.42 ± 12.79 cd
RUH103:pUHR323	6.5 ± 1.2 c	0.052 ± 0.024 ab	19.17 ± 6.65 a	183.98 ± 4.12 a
RUH102:pUHR336- Tn3Hogus-2	4.2 ± 1.5 d	0.007 ± 0.006 fg	2.14 ± 2.32 fg	126.01 ± 9.01 cd
RUH102:pUHR336- Tn3Hogus-4	3.5 ± 1.84 d	0.009 ± 0.006 efg	4.25 ± 4.25 fg	120.82 ± 21.70 d
RUH102:pUHR336- Tn3Hogus-6	4.2 ± 1.79 d	0.019 ± 0.011 def	6.10 ± 5.12 ef	124.34 ± 11.58 cd
Uninoculated (control)	0.0 ± 0.00 e	0.000 ± 0.000 g	0.000 ± 0.00 g	75.72 ± 13.44 e

Means ± S.D. with different letters are significantly different at $p<0.05$

Table 7.5 Nodulation of *Phaseolus vulgaris* by *Rhizobium* TAL1145 *rpoH2* mutants containing the cloned *rpoH2* gene of *Sinorhizobium* BL3 and *Sinorhizobium meliloti* 1021

Strain	Nodule number / plant	Nodule fresh wt (g)/plant	Nitrogenase activity (μmol of ethylene/nodule fresh wt (g)/h)	Plant dry wt (mg)/plant
TAL1145	31.4 ± 3.3 a	0.297 ± 0.069 a	12.96 ± 4.79 a	626.22 ± 58.25 a
RUH102	0.0 ± 0.0 d	0.000 ± 0.000 c	0.00 ± 0.00 b	470.82 ± 144.20 ab
RUH103	0.4 ± 0.5 d	0.000 ± 0.000 c	0.00 ± 0.00 b	401.22 ± 98.19 b
RUH102:pUHR336	35.4 ± 12.0 a	0.297 ± 0.140 a	12.09 ± 5.72 a	505.74 ± 135.50 ab
RUH103:pUHR336	21.6 ± 6.8 bc	0.190 ± 0.081 b	15.70 ± 3.77 a	526.42 ± 84.87 ab
RUH102:pUHR339	20.4 ± 4.1 c	0.200 ± 0.034 b	11.56 ± 4.32 a	430.68 ± 118.94 b
RUH103:pUHR339	21.8 ± 7.5 bc	0.206 ± 0.065 b	14.63 ± 3.95 a	495.64 ± 79.90 ab
RUH102:pUHR323	23.0 ± 9.3 a	0.204 ± 0.069 a	13.97 ± 4.43 a	443.38 ± 51.53 b
RUH103:pUHR323	29.2 ± 10.4 ab	0.228 ± 0.107 ab	13.90 ± 4.58 a	547.46 ± 196.06 ab
RUH102:pUHR336- Tn3Hogus-2	0.4 ± 0.5 d	0.000 ± 0.000 c	0.00 ± 0.00 b	525.56 ± 76.71 ab
RUH102:pUHR336- Tn3Hogus-4	0.2 ± 0.4 d	0.000 ± 0.000 c	0.00 ± 0.00 b	497.48 ± 51.92 ab
RUH102:pUHR336- Tn3Hogus-6	0.4 ± 0.5 d	0.000 ± 0.000 c	0.00 ± 0.00 b	517.78 ± 112.67 ab
Uninoculated (control)	0.2 ± 0.4 d	0.000 ± 0.000 c	0.00 ± 0.00 b	449.10 ± 170.81 b

Means ± S.D. with different letters are significantly different at $p<0.05$

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CHAPTER VIII

OVERALL CONCLUSIONS

This research aimed to develop rhizobial inoculant formulations in liquid form that could reduce the cost of production and provide a quality product and the application convenience farmers desire. Results revealed that cassava, a low cost starch material, could be used for rhizobial inoculant production as a source of carbon for mass cell culture. The first step to convert cassava starch to a useful carbon source is saccharification using amylase-fungi. This process produces glucose and maltose, which were effectively used by rhizobia in genera *Rhizobium, Sinorhizobium, Mesorhizobium* and *Azorhizobium* as carbon sources. Further fermentation of the saccharified starch with yeast produced glycerol. Glycerol proved to be a good carbon source for bradyrhizobia cultivation. In addition, the disrupted yeast cells in the medium provided an additional source of nutrients for bradyrhizobia. Thus, cassava could be an appropriate cheap source of carbon for rhizobial inoculant production using amylase-producing fungus and glycerol-producing yeast.

To investigate new additive substances for liquid inoculant formulation, this research found that many polymers such as, PVP, PEG, PVA, cassava starch, arabic gum and sodium alginate were able to blend with broth medium and used as liquid inoculant instead of peat based inoculant. However, the shelf-life of liquid inoculant was short when stored at room temperature, especially fast-growing strains. Peat-based carrier could maintain the highest number of rhizobial cells on seed stored at

40oC for 48 h, while liquid inoculant containing arabic gum, sodium alginate, PVP, and cassava starch could maintain the number of rhizobial cell on seed at similar level as peat-based inoculant. However, all liquid inoculant formulations promoted nodulation and yield of legume as good as peat-based inoculant.

To use liquid inoculant for Thai soybean varieties, it is important to determine if seeds of these varieties exude any chemicals that may be toxic to rhizobia. This study indicated that the seed of some Thai soybean varieties contained toxic substances that affect growth of rhizobia. However, toxicity varied greatly between soybean varieties and bradyrhizobial strains. Seed exudates of CM2 variety showed a clear effect on bradyrhizobial growth, and this effect was non-specific. The toxin in seed exudates inhibited the growth of rhizobia in other genera as well as *Bradyrhizobium*. There were very small amount of toxic substances presence in Thai soybean seed exudates, owing to the effect of toxin on inhibition could be observed only when the exudates were derived from at least 10 seeds. Moreover, EPS around the cell was one important mechanism for protecting cells from the effect of seed coat toxins. Therefore, Thai soybean seed exudates may not affect the cells of rhizobia in the field condition due to less concentration of toxin and EPS produced by themselves could protect cell from this effect.

Inoculated rhizobia encounter stress conditions after application to seed and planting the seed in soil. Experiments were employed to identify the mechanism within rhizobial cells that allow them to grow when exposed to certain stress conditions. In this study, the adaptive acid tolerance response (ATR) was found in *Sinorhizobium* sp. BL3. The gene involved in ATR has been identified on the basis of enhancing its expression through increasing its copy number in an acid sensitive strain.

The transconjugant containing gene involved in ATR could improve legume symbiosis in acid condition by maintaining higher viability of rhizobia after they are exposed to acid soil conditions. RUH175, derived from transposon-insertion mutagenesis of pUHR324, lost ATR and failed to maintain intracellular pH when cells were exposed to low external pH. Sequencing of the DNA fragment, disrupted by the Tn*3*Ho*gus* insertion led to the identification of *actX*, which encodes a histidine kinase sensor protein of a two-component regulatory system, involved in ATR.

Rhizobial cells are surrounded by a layer of EPS, which has been reported to protect cells under stress conditions. To increase EPS production, it is important to study the regulation of EPS synthesis in rhizobia. The new discovery of transcription factor RpoH2 has role on EPS synthesis and symbiotic functions of *Rhizobium* sp. TAL1145 led to the identification of the function of RpoH2 in *Sinorhizobium* sp. BL3. The cloned *rpoH2* gene of *Sinorhizobium* sp. strain BL3 and of *S. meliloti* restores both EPS and nodulation defects of *rpoH2* mutants of *Rhizobium* sp. strain TAL1145**.** However, the result showed that single mutation of *rpoH2* in BL3 did not involve in the regulation of EPS production and symbiosis with host plant, but *rpoH2* would play an important role in survival during stationary phase or starvation condition.

This research developed rhizobial culture medium with a low-cost available material, and also identified other liquid inoculant formulations which can be used instead of peat-based inoculant. This research also identified possible mechanisms in rhizobia that provide resistance to stress conditions encountered on legume seed or in soil.

APPENDIX

CALCULATION THE COST OF DEVELOPED RHIZOBIAL CULTURE MEDIA PRODUCTION

Calculation the cost of production

Cost of production was composed of materials and chemical substances cost, depreciated cost of instruments, utilities cost, and labor cost.

Table 1 Raw materials and chemical substances cost per 1 liter

Raw materials and chemical substances	Price (Baht)/kg	Price (Baht)/g	Amounts were used (g)	Cost (Baht)
Cassava	1.70	0.0017	200.00	0.34
Potato Dextrose Agar	*5,000.00	5.00	0.78	3.90
Urea	*590.00	0.59	1.00	0.59
KH_2PO_4	*836.00	0.84	1.00	0.84
$MgSO_4.7H_2O$	*450.00	0.45	0.50	0.23
Yeast extract	*5,874.00	5.87	2.50	14.68
$CaCO_3$	*690.00	0.69	3.00	2.07
Total				**22.65**

*Cost of chemical substances were referenced from catalogue of Fluka company (2005)

Table 2 Depreciated cost of instruments (based on 7 years)

Instruments	Price of instrument (Baht)	[a]Cost/hour (Baht)	Time were used (min)	Cost (Baht)
Balance machine	40,000.00	0.65	30	0.33
pH meter	24,992.00	0.41	10	0.07
Shaker	240,000.00	3.91	1,080	70.38/ [b]30 = 2.35
Autoclave	140,000.00	2.28	1	2.28/ [b]2 = 1.14
Total				**3.89**

[a]cost/hour = price of instrument/(7×365×24)

[b]number of materials can be used in one time of using

Utilities cost were composed of electricity cost and water.

Table 3 Electricity costs (1 unit = 4.7411 Baht)

Instruments	Power (kilowatt)	Time were used (hour)	[a]Cost (Baht)
Balance machine	0.045	0.50	0.107
pH meter	0.005	0.17	0.004
Shaker	0.352	18.00	30.04/[b]30 = 1.001
Autoclave	2.000	1.00	9.482/[b]2 = 4.741
Total			**5.853**

[a]Cost = Power (kw)×Time were used (h)×4.7411

[b]number of materials can be used in one time of using

Water cost = 10 Bahts/1,000 liters

Water used 2 liters were cost = (10×2)/1,000 = 0.02 Baht

Then utilities cost = 5.853 + 0.02 = 5.873 Bahts

Labor cost (Baht/h) = salary/(day in one month × working hour)

= 8,000/(30×8) = 33 Bahts/h

Therefore, the cost of production was calculated as indicate in table 4.

Table 4 Total cost of production

List of costs were used for calculated	Cost of production (Baht)
Raw materials and chemical substances cost	22.65
Depreciated cost of instruments	3.89
Utilities cost	5.87
Labor cost	33.00
Total	**65.41**

There were 13 g of glycerol obtained from 1 liter of fermented medium. Therefore, the cost of glycerol production in this study was indicated below.

Cost of glycerol production = 65.41/13 = 5.03 Bahts/g

While, the cost of commercial glycerol was 2.09 Bahts/g (Catalogue of Fluka company, 2005). Therefore, the production of glycerol in this study was expensive than commercial glycerol.

However, fermented medium produced in this study composed of glycerol and yeast cells, which could be used as nutrition sources for rhizobial culture. The results indicated that supplement 5% of fermented medium instead of glycerol and yeast extract in bradyrhizobial culture medium could promote the growth as good as in medium using commercial chemical substances. The calculation of cost for production medium for bradyrhizobia was demonstrated below.

5% of fermented medium were used for supplement. Therefore, 50 ml of fermented medium were used for producing 1 liter of bradyrhizobial medium. As 1 liter of fermented medium cost 65.41 Bahts, therefore 50 ml of fermented medium was equal to (65.41×50)/1000 = 3.27 Bahts. Comparison the cost for producing 1 liter

of medium for bradyrhizobial culturing between the medium prepared by using commercial chemical substances and medium producing by supplement with fermented medium (Table 5). It could be suggested that medium made by supplement with fermented medium was cheaper than medium made by using commercial chemical substances.

Table 5 Cost comparison between the medium prepare by using commercial chemical substances and medium producing by supplement with fermented medium

Chemical substances	Price/g (Baht)	Amounts were used (g)	Cost (Bahts) (Commercial)	Cost (Bahts) (fermented medium)
NH_4Cl	0.97	0.5	0.49	0.49
KH_2HPO_4	0.84	1.0	0.84	0.84
NaCl	0.13	0.2	0.03	0.03
$MgSO_4.7H_2O$	0.45	0.2	0.09	0.09
$CaCl_2$	0.24	0.04	0.01	0.01
$FeCl_3$	0.74	0.04	0.03	0.03
Glycerol	2.09	5.0	10.45	3.27
Yeast extract	5.87	1.0	5.87	
Total			**17.81**	**4.76**

BIBLIOGRAPHY

Miss Panlada Tittabutr was born on December 12, 1978 in Bangkok, Thailand. In 1994, she finished high school from Ratchawinit Bangkeaw School, Samuthprakarn. In 1998, she graduated with the Bachelor's degree of Science in Food Technology from Institute of Agricultural Technology, Suranaree University of Technology, Nakhon Ratchasima. Then, she continued her Doctoral degree in School of Biotechnology, Institute of Agricultural Technology, Suranaree University of Technology, Nakhon Ratchasima and received a scholarship from the Royal Golden Jubilee (RGJ) grant of the Thailand Research Fund in 1999. She had presented posters in the topic of "Development of rhizobial liquid inoculant production: Carbon sources utilization and sugar producing from various raw starch materials by using amylase-producing fungi and yeast fermentation" in World Congress of Soil Science, on August 14th-24th, 2002 in Bangkok, and "Isolation of *Sinorhizobium* sp. BL3 genes that are expressed under low pH" in The 16th CTAHR student research symposium on April 2nd-3rd, 2004 at University of Hawaii, Hawaii. Her works have been published in Biotechnology for Sustainable Utilization of Biological Resources in Tropics**,** in the topic of "Development of rhizobial inoculant production and formulation: Carbon sources utilization and sugar producing from various raw starch materials by using amylase-producing fungi" of volume 15, page 197-200 in 2001, and "Development of rhizobial inoculant production and formulation: Dilution technique and solid state fermentation" of volume 16, page 105-112 in 2002-2003. Moreover, her work has been accepted to publish in World Journal of Microbiology and Biotechnology, in the

topic of “Cassava as a cheap source of carbon for rhizobial inoculant production using an amylase-producing fungus and a glycerol-producing yeast”. Recently**,** she has submitted her works in topic of “A histidine kinase sensor protein gene is necessary for induction of low pH tolerance in *Sinorhizobium* sp. strain BL3” to Antonie van Leeuwenhoek, and another topic of “The cloned *rpoH2* gene of *Sinorhizobium* sp. strain BL3 restores both exopolysaccharide and nodulation defects of *rpoH2* mutants of *Rhizobium* sp. strain TAL1145” to Research in Microbiology.